REMARKS

Claims

Claims 1–5, 17, 21, 33 and 34 are currently pending with claims 6–14, 19, 20, and 23–30 withdrawn from consideration due to election/restriction. Claims 15–16, 18, and 31–32 are cancelled without prejudice or disclaimer.

Rejection under 35 U.S.C. §101 (utility)

Claims 1–5, 17, 21, 22, and 33 stand rejected under 35 U.S.C. §101 as allegedly lacking an apparent or disclosed specific and substantial credible utility. Applicants respectfully traverse this rejection.

The Office Action at page 2 contends that "the specification does not establish specific biological role for the claimed polypeptide." This contention is respectfully traversed. Applicants' specification, for example, the paragraph bridging pages 7 and 8 of the originally-filed specification explicitly teaches that the instant polypeptide "*is* a new [G-protein coupled] receptor for a large glycoprotein ligand originating from testicular secretion." Glycoprotein ligands were known in the art prior to the filing of the instant application. For example, a search in PUBMED database with the term "glycoprotein ligand" resulted in more than 70 scientific publications, all of which were published before the earliest effective date of the instant application (March 13, 1998). A parallel search with the terms "'glycoprotein ligand' AND receptor" resulted in 15 publications published before this date. See, Exhibits A and B. Thus the PTO's quest for ligand of the claimed G-protein receptor, even if valid, is satisfied by the instant disclosure.

Evidentiary scientific publication by Geng et al. (*Journal of Cell Biology*, vol. 137, 743-54, 1997) is directed to the expression of such glycoprotein ligands in the reproductive tissues of animals. According to Geng, P-selectin glycoprotein ligand (PSGL-1), a molecule involved in cell-adhesion, is expressed in the *porcine* zona pellucida (oocyte membrane) and that P-selectin receptor on the acrosomal membrane of *porcine* sperm cells constitutes a receptor for this ligand. See the ABSTRACT and entire RESULTS sections of the article, a copy of which is enclosed herewith for the Examiner's review. Geng thus discloses a novel role of glycoprotein ligands in reproductive physiology, since earlier evidence on the role of the glycoprotein and its cognate receptor were limited to leukocyte function. See, Exhibit B and the enclosed copy of the article by Asa et al.

(*Journal of Biological Chemistry*, vol. 270, 11662, 1995).

In maintaining the utility rejection, the Office Action now contends that a probable utility does not establish practical utility, unless established by actual testing. See, page 5, last paragraph of the Office Action. This contention is clearly misplaced. Amazingly, the present Office Action recites case law (*Bindra v. Kelly* 206 USPQ 570 575), and states that practical utility is established "where utility can be foretold with certainty." See, MPEP §2138.05 Section VIII. However, the Office Action fails to provide any discussion with respect to how the utility requirement can be satisfied via assertion of a practical utility. For example, the Office Action fails to discuss the relevance of the quoted paragraph to the instant case and/or how the asserted use of the claimed ESRP polypeptides in, for example, fertility, is not relevant with respect to the decision in *Bindra*. The Office Action thus presents a very lopsided argumentation on "utility by actual testing" part of the case law, which it seems to be obviously favoring. Applicants respectfully traverse this contention. The law requires that asserted utility be credited.

Applicants' specification explicitly teaches that the polypeptides of the instant invention are "useful in the diagnosis and treatment of male infertility." See, ABSTRACT and page 1, ¶1 of the instant specification. This assertion, coupled with the disclosure contained in Dr. Gottwald's declaration under §1.132, is sufficient to satisfy the statutory requirements under §101. Applicants courteously submit that to maintain the rejection under these circumstances would not only be contrary to the Patent Office's own published standards, but also be blind to the overwhelming scientific evidence regarding the utility of the claimed polypeptides. Accordingly, the rejection under §101 should be withdrawn.

Rejection under 35 USC §112, ¶1 (written description)

The rejection of claims 1–5, 17, 21, 22, and 33 under 35 U.S.C. § 112, first paragraph as allegedly lacking a written description and/or failing to provide enablement is respectfully traversed.

Written description

It is not understood that why this rejection has been maintained. It is submitted that the claims in the current form fully conform to the Written Description Guidelines issued by the USPTO. See, *Synopsis of Written Description Guidelines*, Example 9; *Enzo Biochem. Inc. v. Gen-Probe Inc.*,

63 USPQ2d 1609 (Fed. Cir. 2002). For example, a review of the full content of the specification indicates that an aspect of the claimed invention is the polypeptide which is encoded by a polynucleotide that hybridizes to a complement of SEQ ID NO: 1 under highly stringent hybridization conditions. The encoded protein has a specific, well-established function. The art indicates that hybridization techniques using a known DNA as a probe under highly stringent conditions were conventional in the art at the time of filing. The highly stringent hybridization conditions set forth in the claim yield structurally similar polynucleotides. Thus, a representative number of species is disclosed, since highly stringent hybridization conditions in combination with the coding function of DNA and the level of skill and knowledge in the art are adequate to determine that applicant was in possession of the claimed invention.

Therefore, view of the aforementioned arguments and the explicit disclosure contained in the specification regarding the utility of the claimed polypeptides (for example, as a receptor for a glycoprotein ligand involved in male fertility), it is courteously submitted that Applicants' claims in the current form are in full conformance with the written description guidelines. Withdrawal of the rejection is respectfully requested.

Enablement

With respect to the enablement rejection, Applicants invite the Examiner to review a recent precedential opinion issued by the United States Board of Patent Appeals and Interferences (*Ex parte* Kubin), a copy of which is enclosed herewith.

The facts in Kubin are applicable to the present case. In Kubin, the Examiner contended that "at least 80% identity language" in the absence of any working examples, other than a few representative species, fails to provide enablement of the claimed genus of molecules. See, page 10 of *Ex parte* Kubin. The Examiner alleged that specification did not teach "which 20% . . . of amino acid residues should be changed in order to maintain the biological functions." In response, Appellants argued that the specification disclosed "in detail how to: (1) make variants of SEQ ID NOs: 1 and 2; (2) calculate the percent identity between SEQ ID NOs: 1 and 2 and the variant sequence; and (3) test the variant sequence to determine [functional activity]." See, items 23 and 24 at page 13. Appellants further argued that in view of the high level of skill in molecular biology, methods of making the claimed nucleic acid sequences and screening for activity [were] known in

the art and described in the specification and that the "experimentation involved to produce other sequences within the scope of the claims" and thus to practice the full scope of the claims would have been "well within the skill of those in the art." The amount of experimentation involved would have been routine and not undue. See, items 27–30 at page 14.

The Board of Patent Appeals and Interferences in reversing the enablement rejection concluded:

> "The amount of experimentation to practice the full scope of the claimed invention might have been extensive, but it would have been routine. The techniques necessary to do so were well known to those skilled in the art. *See, e.g., Johns Hopkins Univ. v. Cellpro, Inc.*, 152 F.3d 1342, 1360, 47 USPQ2d 1705, 1719 (Fed. Cir. 1998) ("test [for undue experimentation] is not merely quantitative . . . if it is merely routine"). A "patent need not teach, and preferably omits, what is well known in the art." *Hybritech Inc. v. Monoclonal Antibodies, Inc.*, 802 F.2d 1367, 1384, 231 USPQ 81, 94 (Fed. Cir. 1986). Thus, we conclude the Specification would have enabled the full scope of claim 73. (Emphasis added)

Likewise in the present application, Applicants disclose a genus of GPCR polypeptides having at least 90% sequence identity to a polypeptide sequence with a disclosed primary sequence, along with methods of obtaining other polynucleotide sequences which are commensurate with the claims. In particular, methods of hybridization can be used to isolate sequences which hybridize under stringency conditions set forth in the specification. Conditions (for example, buffer compositions, temperature, and other reagents) which facilitate hybridization are also described. A skilled artisan could routinely utilize translation techniques for identifying polypeptides which are encoded by such hybridizing polynucleotides (for example, using translation tools) and whether such polypeptides would meet the structural element described in Applicants' claims (for example, having at least 90% sequence identity along the entire length to the polypeptide of SEQ ID NO:2). Polypeptide sequences which meet this aspect could then be expressed and assayed for claimed GPCR activity using art techniques, for example, ligand binding studies. The skilled worker may also perform chemical genetic approaches, for example, using anti-sense RNA approaches or knockout techniques, to study receptor function. It would be routine that such polypeptides could be isolated and used by one of ordinary skill in the art using the methods recited in the instant application. Therefore, the level of "experimentation involved to produce other sequences within the scope of the claims" and thus to practice the full scope of the claims would have been "well within

the skill of those in the art."

Rejection under 35 U.S.C. §102 (b) in view of Osterhoff et al.

Claims 1–5, 17, 21, 22, and 31–33 stand rejected under 35 U.S.C. § 102(b) as being allegedly anticipated by Osterhoff et al. (DNA and Cell Biol., vol. 16, pages: 379–389, 1997). Applicants respectfully traverse this rejection.

Osterhoff et al. is the Applicant's own publication, which was published April 1997, i.e., within a year prior to the filing of the parent application (Serial No.: 09/041,745). Applicants submit that the parent application provides an enabling disclosure of the presently claimed invention and provides an adequate written description of the presently claimed invention. Applicants further submit that the parent, like the instant application, meets the requirements of 35 U.S.C. §112, first paragraph, such that benefit of the parent application should be accorded, along with withdrawal of the rejection under 35 U.S.C. §102(b).

In view of the above and attached, it is respectfully submitted that the claims are in condition for allowance. However, should the Examiner have any questions or comments, he is cordially invited to telephone the undersigned at the number below.

The Commissioner is hereby authorized to charge any fees associated with this response to Deposit Account No. 13-3402.

Respectfully submitted,

/Anthony J. Zelano/

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Attorney Docket No.: SCH-2029

Date: February 12, 2008

Encl:

1. Geng et al. (*Journal of Cell Biology*, vol. 137, 743-54, 1997)
2. Asa et al. (*Journal of Biological Chemistry*, vol. 270, 11662, 1995)
3. Exhibits A and B
4. *Ex parte* Kubin (BAPI 2007)

EXHIBIT A

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PMID: 9415550 [PubMed - indexed for MEDLINE]

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Isolated P-selectin glycoprotein ligand-1 dynamic adhesion to P- and E-selectin.

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PMID: 9099943 [PubMed - indexed for MEDLINE]

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Threshold levels of fluid shear promote leukocyte adhesion through selectins (CD62L,P,E)

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Cutaneous lymphocyte antigen is a specialized form of PSGL-1 expressed on skin-homing T cells.
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THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 270, No. 19, Issue of May 12, pp. 11662–11670, 1995
Printed in U.S.A.

The P-selectin Glycoprotein Ligand Functions as a Common Human Leukocyte Ligand for P- and E-selectins*

(Received for publication, November 17, 1994, and in revised form, February 24, 1995)

Darwin Asa‡§, Loretta Raycroft‡§, Li Ma‡§, Paul A. Aeed¶, Paul S. Kaytes‡, Åke P. Elhammer¶, and Jian-Guo Geng‡‖

From ‡Cell Biology and Inflammation Research and ¶Biochemistry Research, Upjohn Laboratories, Kalamazoo, Michigan 49001

P- and E-selectins belong to a family of Ca^{2+}-dependent lectins and function as receptors for myeloid leukocytes. We have described a panel of monoclonal antibodies which recognize a sialoglycoprotein from human neutrophils and HL-60 promyelocytic cells and inhibit adhesion of these cells to P-selectin. In this study, we show that the E-selectin receptor-globulin (E-selectin Rg) affinity chromatography can isolate specifically only one glycoprotein from [^{3}H]glucosamine-labeled HL-60 cells in a Ca^{2+}-dependent manner. This protein has a molecular mass of ~120 kDa under reducing conditions, which appears to be identical with the previously characterized glycoprotein ligand for P-selectin. The molecule can be cross-depleted by and cross-bound to the E- and P-selectin columns. The chromatographic profile of desialylated *O*-linked carbohydrates from molecules purified by P- and E-selectin affinity chromatography are identical. Both have five structures at 12.8, 9.8, 6.3, 3.5, and 2.5 glucose units. PL5 monoclonal antibody to the P-selectin sialoglycoprotein ligand, E-selectin Rg, and antiserum to P-selectin glycoprotein ligand-1 (PSGL-1) all recognize the purified P-selectin ligand on ligand blots and immunoblots. Furthermore, PL5 monoclonal antibody blocks adhesion of HL-60 cells and human neutrophils to E-selectin Rg. Taken together, our results demonstrate that the P- and E-selectin ligand defined in this study is PSGL-1 and suggest that this molecule is an important leukocyte ligand for both P- and E-selectins.

P-selectin (CD62P) and E-selectin (CD62E) are members of the selectin family of leukocyte adhesion molecules (1–5). Both P- and E-selectins mediate leukocyte adhesion to endothelial cells under vascular shear stress in capillary venules. P-selectin also mediates rosetting of activated platelets onto leukocytes (1–5).

P-selectin has been shown to bind to an ~240-kDa homodimeric sialoglycoprotein (~120 kDa under reducing conditions) from myeloid cells with high affinity (6–8). Recently, a mucin-like transmembrane protein, called PSGL-1,[1] has been cloned (9). COS cells co-transfected with PSGL-1 and α(1,3/4)fucosyltransferase cDNAs express a homodimer of ~240 kDa and support leukocyte adhesion in a P-selectin-dependent manner. We have previously reported the development of a panel of monoclonal antibodies which recognize an ~240-kDa molecule from human neutrophils and HL-60 cells and inhibit adhesion of these cells to P-selectin (10). These results strongly suggest that the 240-kDa molecule is a leukocyte ligand for P-selectin.

A number of leukocyte ligands from various species have been described for P- and E-selectin. E-selectin, but not P-selectin, recognizes an ~150-kDa monomeric glycoprotein from mouse myeloid cells metabolically labeled with [^{35}S]methionine and [^{35}S]cysteine (11, 12). This binding is Ca^{2+}-dependent and can be blocked by an antibody to mouse E-selectin. In contrast, the mature mouse neutrophils contain glycoproteins of 230 kDa and 130 kDa, which bind to both P- and E-selectins in a Ca^{2+}-dependent, sialidase-sensitive fashion (12). A single glycoprotein (250 kDa under nonreducing conditions and 280 kDa under reducing conditions), isolated from bovine γ/δ T cells, binds to human E-selectin in a Ca^{2+}-dependent, sialidase-sensitive manner (13). Furthermore, P-selectin, but not E-selectin, recognizes an ~160-kDa monomeric glycoprotein from mature mouse neutrophils and human HL-60 cells (12).

There are some experimental results which suggest that the P-selectin glycoprotein ligand might function as a ligand for E-selectin. First, the conditioned medium from COS cells co-transfected with both the truncated soluble form of PSGL-1 and α(1,3/4)fucosyltransferase cDNAs mediates adhesion to radiolabeled CHO-P-selectin cells and CHO-E-selectin cells. In both cases, the cell adhesion is Ca^{2+}-dependent and inhibitable with respective neutralizing antibodies (9). Second, ^{125}I-PSGL-1 from human neutrophils binds directly to truncated soluble forms of P- and E-selectins in a Ca^{2+}-dependent manner (8). However, the binding affinity for E-selectin (IC_{50} ~ 25 μM) is at least 50-fold weaker than that for P-selectin (IC_{50} ~ 0.5 μM).

Taken together, these results suggest that E-selectin recognizes several glycoproteins with different molecular masses including ~130-kDa, ~150-kDa, and ~230-kDa monomeric molecules in mouse neutrophils, an ~250-kDa monomeric molecule from bovine γ/δ T cells, and an ~240-kDa dimeric molecule (P-selectin glycoprotein ligand) from human neutrophils and HL-60 cells (8, 11–13). However, it is not known whether the P-selectin glycoprotein ligand is the only high affinity ligand recognized by E-selectin in human neutrophils and HL-60 cells. Moreover, the biological importance of the P-selectin glycoprotein ligand on the cell surface of human neutrophils and HL-60 cells as a ligand in E-selectin-mediated leukocyte adhe-

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§ The first three authors contributed equally to this study.

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[1] The abbreviations used here are: PSGL-1, P-selectin glycoprotein ligand-1; P/ESGL-1, P- and E-selectin glycoprotein ligand-1; Rg, receptor-globulin; mAb, monoclonal antibody; PBS, phosphate-buffered saline at pH 7.4; PAGE, polyacrylamide gel electrophoresis; IgG, immunoglobulin G; BSA, bovine serum albumin; SLe^x, Siaα2–3Galβ1–4(Fucα1–3)GlcNAc; HBSS, Hank's balanced salt solution with $CaCl_2$ and $MgCl_2$.

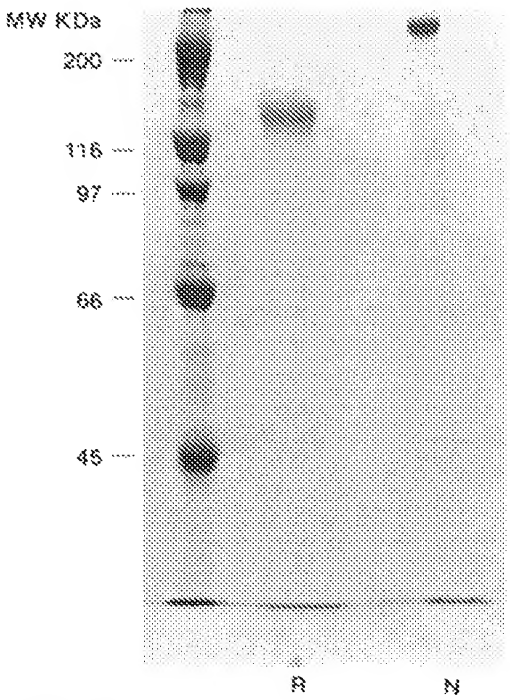

FIG. 1. **Purity of E-selectin Rg.** The construction, expression, and purification of E-selectin Rg were carried out as described under *Proteins* under "Experimental Procedures." Samples of the purified E-selectin Rg (10 μg/lane) were boiled for 5 min in the presence (*R*) or absence (*N*) of 2% β-mercaptoethanol (v/v) and analyzed by 7% SDS-PAGE and Coomassie Blue staining.

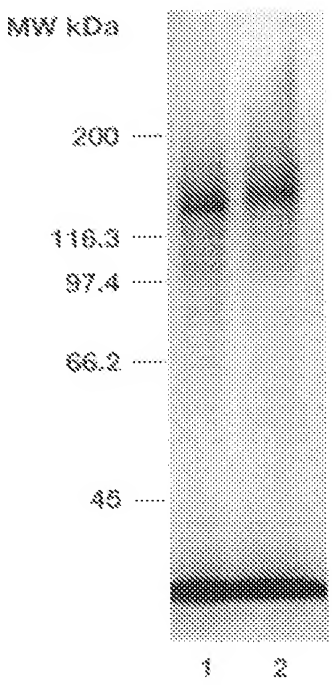

FIG. 2. **Purification of glycoprotein ligands for P- and E-selectin.** HL-60 cells were metabolically labeled with [^{3}H]glucosamine, and the ligand molecules were purified from the P-selectin affinity column or from the E-selectin Rg affinity column with EDTA elution as described previously (10). The radioactive fractions of EDTA eluates from each column were pooled together. After dialysis, samples were boiled for 5 min in the presence of 2% β-mercaptoethanol (v/v) and then subject to 7% SDS-PAGE followed by autoradiography. *Lane 1*, the eluates from the P-selectin affinity column; *lane 2*, the eluates from the E-selectin Rg affinity column.

sion remains unclear.

In the present study, we show that only one radiolabeled glycoprotein is purified specifically by E-selectin Rg affinity chromatography. The molecular mass and *O*-linked carbohydrate profile of this molecule are identical with those of the P-selectin glycoprotein ligand isolated by P-selectin affinity chromatography. This molecule is recognized by PLδ mAb, E-selectin Rg, and PSGL-1 antiserum. Furthermore, PLδ, a monoclonal IgM antibody raised against the P-selectin ligand (10), also blocks adhesion of human neutrophils and HL-60 cells to E-selectin. Thus, our results suggest that this glycoprotein is an important human leukocyte ligand for both P- and E-selectins.

EXPERIMENTAL PROCEDURES

Materials—DIG Glycan Detection Kit and *Arthrobacter ureafaciens* sialidase (EC 3.2.1.18) were purchased from Boehringer Mannheim. BBA1 mAb to E-selectin was obtained from R&D Systems. Purified human IgG was purchased from Sigma. Leu-8 mAb to L-selectin was obtained from Becton Dickinson. ImmunoPure IgG Orientation Kit and Micro BCA Kit for measurement of protein concentrations were purchased from Pierce. Affi-Gel 15 Sepharose was obtained from Bio-Rad. Dialysis membrane tubing (12-kDa to 14-kDa molecular mass cut-off) was purchased from Spectra-Pore and used throughout this study. [^{3}H]Glucosamine (20–40 Ci/mmol) was purchased from Amersham or DuPont NEN. SepPak C_{18} cartridges were obtained from Waters. Superdex 200 column and Smart System were purchased from Pharmacia Biotech Inc. All other chemicals were purchased as described previously (10).

Proteins—P-selectin was purified from outdated human platelets as published (11). P-selectin Rg was prepared as described previously.[2]

E-selectin Rg (the lectin-epidermal growth factor-repeat domains 1 to 6 of E-selectin exoplasmic portion fused with the hinge, C_H2, and C_H3 domains of human IgG1) was constructed as described (15).[2] The establishment of a stable CHO cell line secreting E-selectin Rg, the serum-free tissue culture of this CHO cell line, and the purification of E-selectin Rg were exactly carried out as described.[2] The biotinylated BBA1 mAb to E-selectin was used (instead of biotinylated P7 mAb to P-selectin) for sandwich enzyme-linked immunosorbent assay to screen the high secreting clones. The purified E-selectin Rg was dialyzed against PBS, and samples were subject to SDS-PAGE followed by Coomassie Blue staining, as shown in Fig. 1. E-selectin thus prepared mediated a Ca^{2+}-dependent adhesion of HL-60 cells and human neutrophils (see below). The sample of E-selectin Rg was analyzed by NH_2-terminal amino acid sequencing with following sequence: WSYGT-STEAM TYDEASAYXQ QKYTHXVAIQ NS (X: ambiguous residue), fully consistent with the protein sequence of human E-selectin (15). The $E^{1\%}_{280}$ for E-selectin Rg is 16.3 calculated according to the results of acid hydrolysis and compositional analysis of amino acids.

Antibodies—Monoclonal IgG antibodies against P-selectin including an adhesion-blocking mAb, P7, an adhesion nonblocking mAb, P35, and a monoclonal IgM antibody against a P-selectin glycoprotein ligand, PLδ, were prepared and characterized as described (10). Rabbit antiserum to PSGL-1 was prepared by hyperimmunization of the rabbit with purified recombinant PSGL-1 receptor-globulin. This antiserum was kindly provided by Dr. Dietmar Vestweber (Hans Spemann Laboratory at the Max Planck Institute for Immunology, Germany). CSLEX monoclonal IgM antibody to SLe^x was purified from mouse ascites as described previously (10).

The purified E-selectin Rg was used as the antigen in primary and booster immunization with BALB/c mice as described (10). Twelve weeks later, the spleen cells of the two mice were fused with myeloma cells. The hybridomas were screened for recognition of E-selectin Rg using an enzyme-linked immunosorbent assay (17, 18). As the immunogen, E-selectin Rg, contained a Fc fragment (without the C_H1 domain) of human IgG1, the hybridoma supernatants were preincubated with human IgG at 37 °C for 1 h (0.1 mg/ml final concentration of human IgG) before transferring them into the microtiter wells immobilized with E-selectin Rg to avoid generation of antibodies against the heavy chain of human IgG1. After subcloning twice, we obtained a panel of four hybridomas (E1, E2, E3, E4, designated as E for E-selectin).

The ascites from individual hybridomas were prepared for purification of antibodies using Protein A chromatography (19). Each of these

[2] L. Ma, K.-C. Chen, P. S. Kayton, B. A. Leone, C. F. Tormey, D. J. Staples, M. S. Balcarek, H. D. Fischer, C. W. Smith, and J.-G. Gong, submitted for publication.

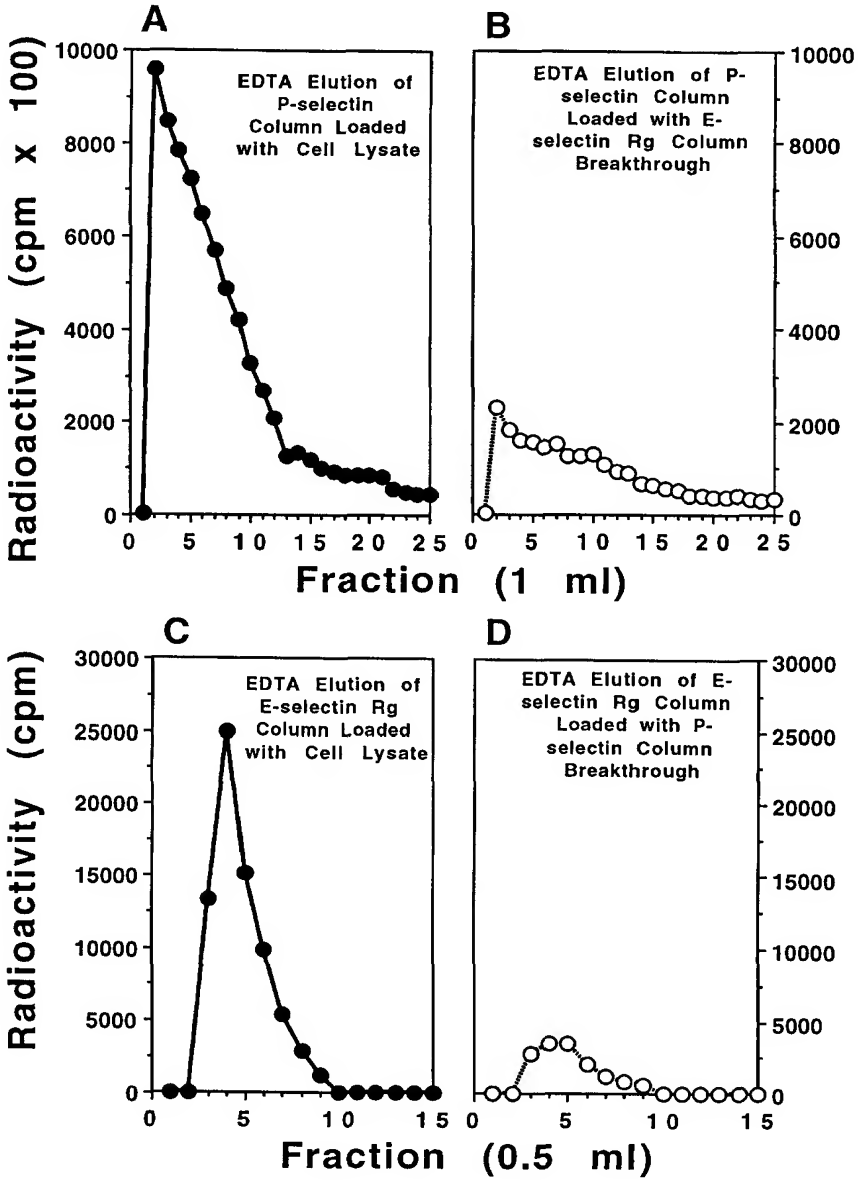

FIG. 3. **Cross-depletion of P- and E-selectin ligands.** Two equal amounts of the labeled HL-60 cell lysates were applied to the P-selectin affinity column and the E-selectin Rg affinity column in the presence of Ca^{2+}. After extensive washing, the bound ligands were eluted with EDTA from the P-selectin affinity column (*A*) and from the E-selectin Rg affinity column (*C*). The breakthrough of the E-selectin Rg column was reapplied to the P-selectin column while the breakthrough of the P-selectin column was reapplied to the E-selectin Rg column. After washing, the bound ligands were again eluted with EDTA from the P-selectin column (*B*) and from the E-selectin Rg column (*D*). The radioactivities of the sample loading and column washing were omitted. The radioactivities of each fraction from EDTA elution were plotted.

antibodies recognized E-selectin Rg but did not recognize P-selectin Rg or human IgG in an enzyme-linked immunosorbent assay (data not shown). They also bound to CHO cells expressing full-length E-selectin but did not bind to CHO cells expressing full-length P-selectin or thrombin-activated platelets using a flow cytometric assay (data not shown). Furthermore, these antibodies did not recognize Jurkat T-lymphocytes whereas Leu-8 mAb to L-selectin did recognize these cells (data not shown).

Affinity Purification of Radioactive Ligands—HL-60 cells were metabolically labeled with [^{3}H]glucosamine (50 μCi/ml) in RPMI medium containing 10% fetal calf serum for 48 to 72 h as described previously (10).

The P-selectin affinity column was prepared by coupling 10 mg of platelet P-selectin directly onto 10 ml of Affi-Gel 15 Sepharose (10). The E-selectin Rg affinity column was prepared by cross-linking 5 mg of E-selectin Rg onto 2 ml of Protein A resin by dimethyl pimelimidate according to the recommendations of the ImmunoPure IgG Orientation Kit (Pierce). A BSA-Sepharose column (10-ml resin) was used as a guard column for P-selectin and E-selectin Rg affinity columns. For specificity control, the human IgG columns were prepared by cross-linking 10 mg of human IgG onto 2 ml of Protein A resin for each column prepared exactly as the E-selectin Rg column described above.

The glycoprotein ligands were purified using an EDTA elution of P-selectin affinity column or E-selectin Rg affinity column as published previously (10). The eluted fractions were pooled together and separately placed into dialysis tubing which had been treated with 0.1% BSA (w/v) to lower nonspecific binding. Samples were dialyzed against H_2O at 4 °C overnight. After dialysis, they were frozen in a dry ice/acetone bath and lyophilized in glass tubes to dryness. The lyophilized samples were redissolved in a small volume of PBS, and the radioactivities were counted using a Packard liquid scintillation counter (low level detection). This one-step affinity purification could yield P- and E-selectin ligands radiochemically pure as assessed by SDS-PAGE and autoradiography.

Cross-depleting Experiments—To demonstrate cross-depletion of the glycoprotein ligands for P- and E-selectins, the [^{3}H]glucosamine-labeled cell lysates from 2×10^7 cells (labeled with 20 mCi of isotope) were prepared as described above. Two equal aliquots of the cell lysates were applied to the BSA guard column first and then separately applied to P- and E-selectin columns. After washing, the bound materials were eluted from the P-selectin or E-selectin Rg affinity column as described above. The breakthrough from the P-selectin affinity column was reapplied to the E-selectin Rg affinity column, while the breakthrough from the E-selectin Rg affinity column was reapplied to the P-selectin affinity column. After washing, the bound materials were eluted with EDTA as above. Aliquots of each eluted fraction were counted, and radioactivities of these fractions were plotted. The radioactive fractions were pooled, dialyzed, and analyzed by 7% SDS-PAGE and autoradiography as above.

Cross-binding Experiments—The cross-binding properties of the ~120-kDa molecule on P- and E-selectin affinity columns were demonstrated as follows. The radioactive ligands were purified separately

from [^{3}H]glucosamine-labeled HL-60 cells to apparent homogeneity by P- or E-selectin affinity chromatography as above (data not shown). The purified ligands were dialyzed individually into the buffer containing 1 mM $CaCl_2$. The E-selectin column-purified ligand (10,000 cpm) was reapplied to the P-selectin column, while the P-selectin column-purified ligand (10,000 cpm) was re-applied to the E-selectin column. After washing, the bound ligands were eluted with EDTA from these columns and the radioactivities were counted and plotted. For specificity control, the purified P- and E-selectin ligands (10,000 cpm for each ligand molecule) were separately loaded onto human IgG columns. Following washing, the radioactivities of the EDTA elution fractions were counted.

Release of O-linked Sugars by β-Elimination—The radiochemically pure glycoprotein ligands isolated by the P- or E-selectin columns were precipitated separately with methanol/chloroform (19) to remove salts and detergent. Following evaporation to dryness, the O-linked oligosaccharides were released by mild alkaline sodium borohydride treatment (20). The released oligosaccharides were dissolved in H_2O and washed through SepPak C_{18} cartridges to remove polypeptide fragments.

Desialylation of Oligosaccharides—The released O-linked oligosaccharides were digested with *A. ureafaciens* sialidase (2 units/ml) in 100 mM acetate buffer, pH 5.0, for 18 h.

Carbohydrate Profiles—The desialylated O-linked oligosaccharide "fingerprints" from the molecules isolated on the P- and E-selectin columns were obtained by separating the desialylated oligosaccharides on size exclusion chromatography (GlycoMap Chromatograph, Oxford GlycoSystems). A Bio-Gel P-4 type column (Glycan Sizing Column, Oxford GlycoSystems) and the high resolution program (50 μl/min for 366 min followed by a linear increase to 200 μl/min over 234 min) were used. Two drop fractions (approximately 90 μl) were collected and counted.

Affinity Purification of P-selectin Ligand from Unlabeled HL-60 Cells—To obtain biochemical pure P-selectin ligand from unlabeled HL-60 cells, the preparation of membrane lysates from ~50 liters of HL-60 cells and the PL5 antibody affinity chromatography were carried out as described previously (10). These materials were dialyzed against PBS, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 0.01% Brij-35 (v/v) and 0.02% N_3Na (w/v) (Buffer A) at 4 °C overnight. After a brief centrifugation (14,000 rpm for 10 min at 4 °C), the clear supernatant was loaded onto the P-selectin affinity column as described (10). After washing the P-selectin affinity column with 50 ml of Buffer A, the bound proteins were eluted with PBS, 10 mM EDTA, 0.01% Brij-35 (v/v), and 0.02% NaN_3 (w/v) (Buffer B).

Size Exclusion Chromatography—For the purpose of purification and characterization, the nonradioactive P-selectin ligand was dialyzed against H_2O and lyophilized as above. The lyophilized material was then resuspended in 200 μl of PBS, 0.01% Brij-35 (v/v), and 0.02% N_3Na (w/v) (Buffer C). Following another brief centrifugation, a 50-μl aliquot was injected into a 100-μl sample loop for each run on Superdex 200 size exclusion chromatography, using Buffer C at 40 μl/min at 22 °C and collection as 50-μl fractions (Smart System, Pharmacia). The protein peaks were monitored at $A_{206\ nm}$ and $A_{280\ nm}$. The radiochemical pure P-selectin ligand was chromatographed exactly as above. A 5-μl aliquot of each P-selectin ligand fraction was counted for radioactivity. The counting numbers were then multiplied by 10 and plotted.

Ligand Blotting and Immunoblotting—Aliquots of the protein peak from Superdex 200 chromatography (1 μg/lane) were electrophoresed and transferred to a polyvinylidene difluoride membrane (Immobilon-P, Millipore). The membranes were separately probed with E-selectin Rg and PL5 IgM mAb (both at 1 μg/ml) as well as PSGL-1 antiserum (1:100 dilution of serum). The membranes were also probed with human IgG (10 μg/ml) and nonimmunized serum (1:10 dilution of serum) for experimental control. For detection, the membranes were individually incubated with biotinylated P33 mAb, goat anti-mouse IgM antibody, and goat anti-rabbit and anti-human IgG antibodies (all at 5 μg/ml) followed by a streptavidin-peroxidase complex (Vectastain ABC Kit, Vector Laboratories) and a chemiluminescent detection system (Amersham) as described previously (10).

Cell Adhesion Assay—E- and P-selectin Rgs and human IgG were diluted in HBSS (3 μg/ml) and immobilized onto the 96-well microtiter plates (50 μl/well) at 4 °C overnight. The cell adhesion assay was performed exactly as described previously (21).

Protein Concentration Measurement—Protein concentrations were measured using Micro BCA Assay with BSA as standard (Pierce).

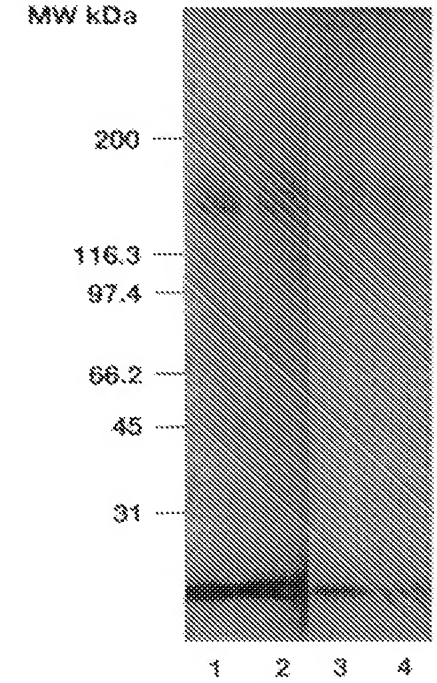

FIG. 3. **Purity of the cross-depleted P- and E-selectin ligands.** To ensure the purity of P- and E-selectin ligands in the cross-depleting experiment, all EDTA eluates from P- and E-selectin affinity columns were separately pooled. Aliquots of each pool (50,000 cpm) were mixed with SDS sample dye in the presence of 5% β-mercaptoethanol (v/v) and boiled for 5 min. Samples were then fractionated on 7% SDS-PAGE followed by autoradiography. *Lane 1*, the eluate of the P-selectin column after loading HL-60 cell lysate; *lane 2*, the eluate of the P-selectin column after loading the breakthrough from E-selectin Rg column onto the P-selectin column. *lane 3*, the eluate of the E-selectin Rg column after loading HL-60 cell lysate; *lane 4*, the eluate of the E-selectin Rg column after loading the breakthrough from the P-selectin column onto the E-selectin Rg column.

RESULTS

P- and E-selectin Both Recognize ~120-kDa Ligands—An ~120-kDa glycoprotein (under reducing conditions) can be isolated from [^{3}H]glucosamine-labeled HL-60 cells by affinity chromatography using platelet P-selectin or the PL5 mAb (6–8, 10). This molecule from the membrane extracts of human leukocytes and HL-60 cells is also recognized by P-selectin and PL5 mAb by ligand blotting and immunoblotting (6–10). In this study, we have constructed, expressed, and purified E-selectin Rg to be used as a functional equivalent for E-selectin. Following the same approach of affinity purification as that for P-selectin ligand isolation, we investigated how many and which molecule(s) can be isolated from [^{3}H]glucosamine-labeled HL-60 cells by E-selectin Rg affinity chromatography. As shown in Fig. 3, E-selectin Rg affinity chromatography specifically isolated only one glycoprotein (*lane 2*) from labeled human HL-60 cells. Surprisingly, this molecule had a molecular mass of ~120 kDa under reducing conditions; *i.e.* it has a molecular mass identical with that of the P-selectin glycoprotein ligand isolated by P-selectin affinity chromatography (*lane 1*). The purified ligands from both P- and E-selectin affinity chromatography routinely account for less than 0.001% of total radioactivity from the cell lysates which have been applied to the affinity columns (data not shown). As an experimental control for specificity, the lysates of labeled HL-60 cells were also applied to human IgG affinity column in the presence of Ca^{2+}. After washing, EDTA elution of this column yielded an insignificant amount of radioactivity which could not be precipitated by methanol/chloroform (19), suggesting that it probably represented free [^{3}H]glucosamine instead of glycoproteins

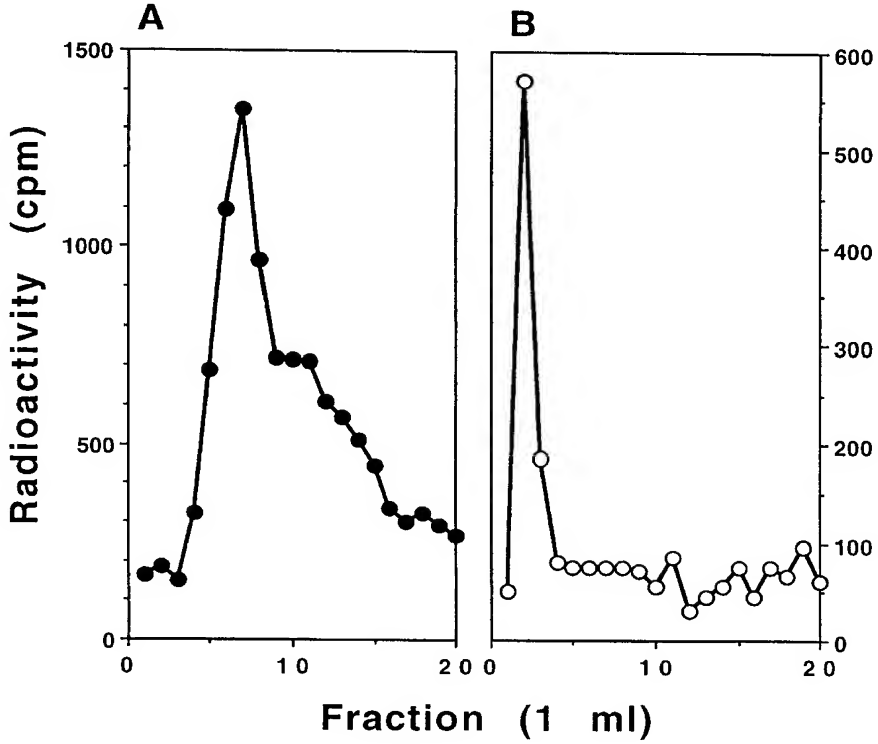

FIG. 5. **Cross-binding of P- and E-selectin ligands.** The radioactive ligands were isolated from the [^{3}H]glucosamine-labeled HL-60 cells by P- and E-selectin affinity chromatography as above. The purified E-selectin ligand was reapplied to the P-selectin column. After washing, the bound ligand was eluted with EDTA, and the radioactivities of EDTA elution fractions were counted (*A*). Similarly, the purified P-selectin ligand was reapplied to the E-selectin column, and the bound ligand was eluted with EDTA (*B*).

(data not shown).

Cross-depletion of P- and E-selectin Ligands—As P- and E-selectins both bind to the ~120-kDa molecules, we investigated whether these ligands might actually be the same molecule. To answer this question, cross-depletion experiments were carried out. Two equal amounts of lysates from [^{3}H]glucosamine-labeled HL-60 cells were applied separately to the P- or E-selectin affinity column in the presence of Ca^{2+}. After washing, the bound ligands were eluted with EDTA from the P- (Fig. 3*A*) and E-selectin columns (Fig. 3*C*). An aliquot of each fraction from EDTA elution was counted and plotted for radioactivity. As the bound radioactivities accounted for less than 0.001% of total sample radioactivities, only the EDTA-eluted radioactivities were presented. The radioactivities from the sample loading and column washing were omitted.

The collected breakthrough from the E-selectin Rg chromatography was pooled together and reapplied to the P-selectin affinity column while the collected breakthrough from the P-selectin chromatography was pooled together and reapplied to the E-selectin affinity column. After extensive washing, the bound ligands were again eluted with EDTA from the P-selectin column (Fig. 3*B*) and E-selectin column (Fig. 3*D*). The radioactivities were counted and the EDTA-eluted fractions were plotted. Together, these experiments demonstrated that passing the cell lysates through the P-selectin affinity column depleted the majority of their binding to the following E-selectin affinity column (Fig. 3, *A* and *D*) and vice versa (Fig. 3, *C* and *B*). However, it was noticed that passing the cell lysates through either the P- or E-selectin affinity column could not completely deplete their binding to the following E-selectin column (Fig. 3*D*) or P-selectin column (Fig. 3*B*). As the similar amounts of ligand binding were also observed when repeating the same (not cross-over) P- or E-selectin affinity chromatography (data not shown), we believed that the incomplete depletion of P- and E-selectin ligands from labeled HL-60 cells were most likely due to the nature of the P- and E-selectin affinity chromatography.

To ensure the purities of each EDTA eluate from P- and E-selectin affinity columns, they were fractionated on SDS-PAGE and visualized by autoradiography (Fig. 4). All eluates showed radioactive bands only at ~120 kDa under reducing conditions, confirming the identity of the molecules binding to the affinity columns in the cross-depletion experiments. Therefore, our results suggest that the same glycoprotein molecule of ~120 kDa is probably recognized by both P- and E-selectin.

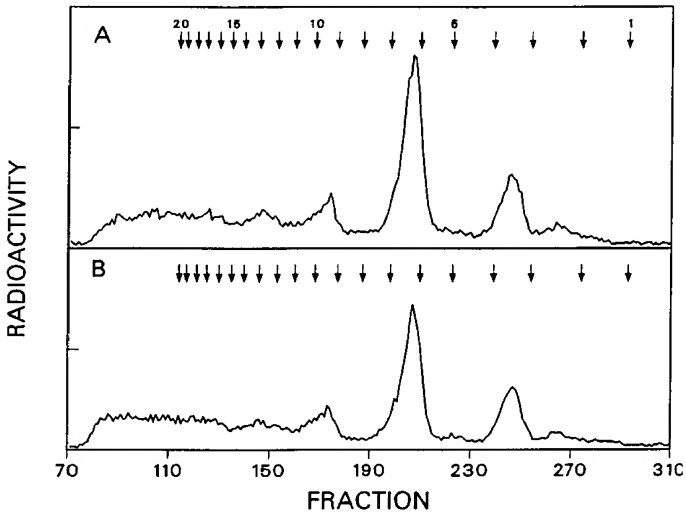

FIG. 6. **Carbohydrate chromatographic profiles of P- and E-selectin ligands.** The *O*-linked carbohydrates from the purified P- and E-selectin ligands were chemically released by β-elimination (20). After desialylation, the chromatographic profiles of the *O*-linked oligosaccharides were obtained by separating them on a size exclusion column (Glycan Sizing Column, Oxford GlycoSystems) and scintillation counting of each fraction. *A*, fingerprints of the *O*-linked oligosaccharides from P-selectin ligand. *B*, fingerprints of the *O*-linked oligosaccharides from E-selectin ligand. *Arrows* are standards for glucose units (Oxford GlycoSystems).

Cross-binding of P- and E-selectin Ligands—To further demonstrate that both P- and E-selectins recognize the same ligand

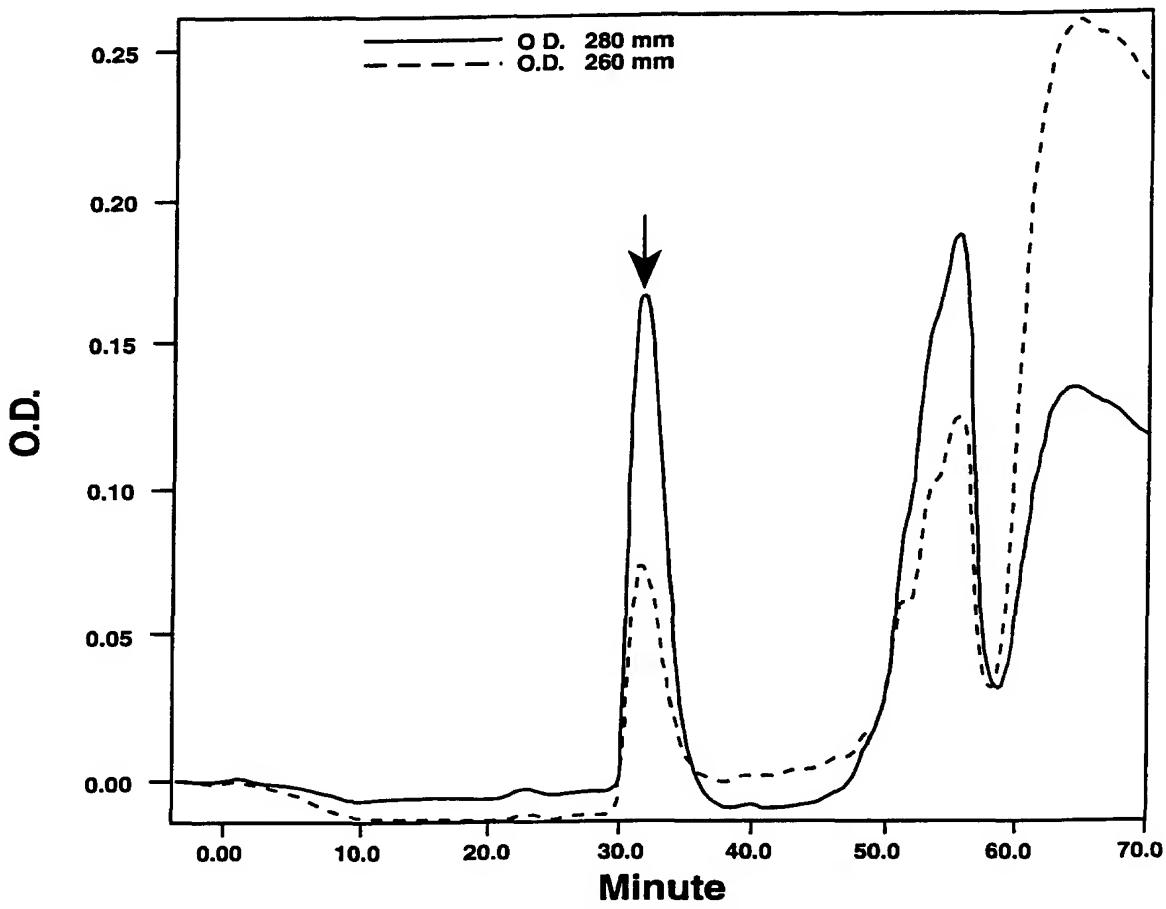

FIG. 7. **Isolation of unlabeled P-selectin ligand.** The P-selectin ligand from unlabeled HL-60 cells was isolated by PL5 mAb chromatography and P-selectin affinity chromatography as described under "Experimental Procedures." The eluate from P-selectin affinity column shows a distinctive protein peak (the ratio of $A_{280\ nm}$ over $A_{260\ nm}$ about 2:1) on a Superdex 200 column (Smart System, Pharmacia). It is identical with the radioactive peak of the P-selectin ligand purified from labeled HL-60 cells (*arrow*).

with high affinities, a cross-binding experiment was carried out. The binding of E-selectin-purified ligand to the P-selectin column could be demonstrated by EDTA elution of the P-selectin column (Fig. 5*A*). Similarly, the binding of P-selectin-purified ligand to the E-selectin column could be demonstrated by EDTA elution of the E-selectin column (Fig. 5*B*). For experimental control, the P- and E-selectin-purified ligands were separately loaded onto the human IgG columns. After washing, they were eluted with EDTA. The insignificant amounts (less than 100 cpm from each column) were obtained in EDTA elution fractions (data not shown). These results suggest the specificity of the cross-binding experiments. Thus, our data strongly support that both P- and E-selectin recognize the same ~120-kDa molecule as their high affinity ligands.

Determination of Carbohydrate Profiles—The biological importance of the *O*-linked carbohydrates as selectin ligands has been reported in numerous publications (7–9, 22–25). To confirm the identity of the glycoprotein ligands, we compared the chromatographic profiles of the desialylated *O*-linked carbohydrates released from the ligand molecules purified by P- and E-selectin affinity chromatography. The *O*-linked oligosaccharides were released by β-elimination as described (20). After desialylation with *A. ureafaciens* sialidase, the oligosaccharides were separated on a Glycan Sizing Column (Oxford GlycoSystems), and the radioactivity was determined in each fraction. As shown here, the fingerprint of the *O*-linked carbohydrates from the P-selectin ligand (Fig. 6*A*) was identical with that from the E-selectin ligand (Fig. 6*B*). Both had five identifiable structures with relatively similar percentages of radioactivity for each peak eluting in the positions of oligomers composed of 12.8, 9.8, 6.3, 3.5, and 2.5 glucose units, respectively. These results indicate that the P- and E-selectin glycoprotein ligands may contain identical *O*-linked oligosaccharides.

Recognition of the P-selectin Ligand by PL5 mAb, E-selectin Rg, and PSGL-1 Antiserum—Recently, Moore *et al.* (8) showed that the ligand molecules isolated by P-selectin affinity chromatography could be precipitated by antisera to synthetic peptides corresponding to the amino acid sequence of PSGL-1. To ascertain the immunological identity of the ligand molecules in our hands, we have purified the P-selectin ligand from unlabeled HL-60 cells by a sequential isolation procedure involving PL5 antibody affinity chromatography, P-selectin affinity chromatography, and size exclusion chromatography on a Superdex 200 column (Smart System, Pharmacia) (Fig. 7). Interestingly, this molecule (20 μg/lane) was not detectable by silver staining (Fig. 8*A*, *lane 2*). However, it could be stained by Glycan Stain (Fig. 8*A*, *lane 3*), a method in which adjacent hydroxyl groups in glycoprotein oligosaccharides are oxidized to aldehyde groups by mild periodate treatment. Digoxigenin is then covalently attached to these aldehydes via a hydrazide group. Detection is accomplished by a digoxigenin-specific antibody coupled with alkaline phosphatase. As shown in Fig. 8*B* (*lanes 1–4*), the purified P-selectin ligand is recognized by PL5 mAb, E-selectin Rg, and PSGL-1 antiserum. The molecule is not recognized by human IgG and nonimmunized serum in a parallel control experiment (data not shown). Therefore, these results confirm the immunological identity of the molecule recognized by P-selectin, E-selectin Rg, and PL5 mAb as PSGL-1.

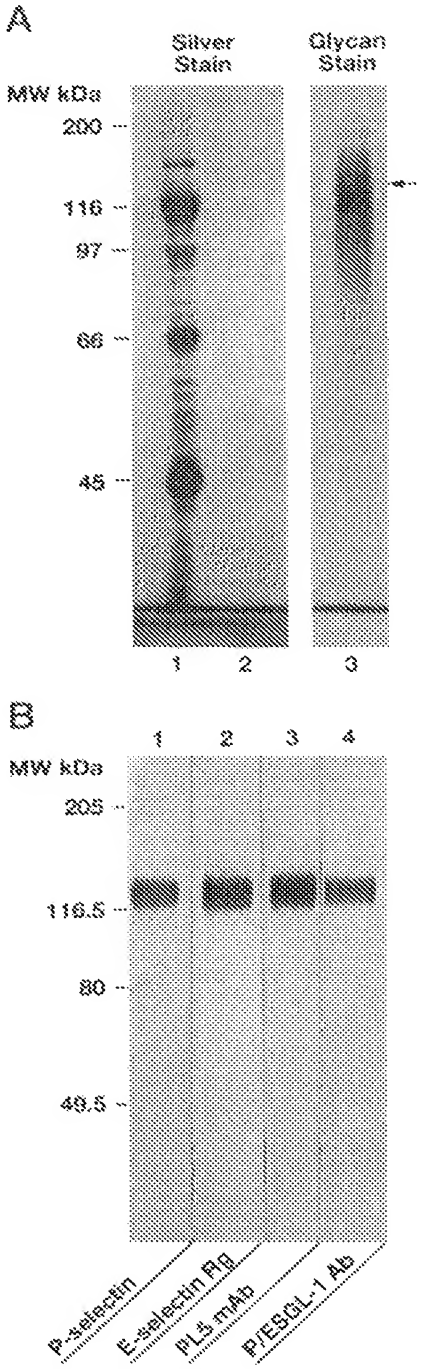

FIG. 8. **Recognition of the purified P-selectin ligand by PL5 mAb, E-selectin Rg, and PSGL-1 antiserum.** The protein peak from size exclusion chromatography was subject to 7% SDS-PAGE under reducing conditions. The protein was either silver-stained (20 μg/lane) (*A, lane 2*) or transferred onto a polyvinylidene difluoride membrane. The P-selectin ligand at ~120 kDa (10 μg/lane) was detected with DIG Glycan Detection Kit for glycoprotein staining (*A, lane 3*). No proteins were visible with silver staining, suggesting that this molecule was inaccessible to silver staining and that there were no detectable contaminations (*A, lane 2*). The polyvinylidene difluoride membranes (1 μg/lane) were also probed with P-selectin (*B, lane 1*), E-selectin Rg (*B, lane 2*), PL5 mAb (*B, lane 3*), and P/ESGL-1 antiserum (*B, lane 4*). See "Experimental Procedures" for details of the detection methods.

PL5 mAb Inhibits Adhesion of HL-60 Cells to E-selectin—We have previously reported that a panel of mAbs to a P-selectin ligand inhibits adhesion of human neutrophils and HL-60 cells to P-selectin (10). In this study, we have shown that this ligand is also recognized by E-selectin Rg. Then, the question is whether these mAbs also abrogate leukocyte adhesion to E-selectin. To answer this question, a cell adhesion assay was carried out using P- and E-selectin Rgs immobilized on 96-microtiter wells as described previously (21).[8] As shown in Fig. 9, PL5 mAb specifically attenuated adhesion of HL-60 cells to both P- and E-selectin Rgs. This indicates that the PL5 mAb raised against a P-selectin glycoprotein ligand is also able to block adhesion of HL-60 cells to E-selectin. The binding of HL-60 cells in this assay was specific. The adhesion of HL-60 cells was divalent cation-dependent. It was inhibited by respective neutralizing mAbs (P7 to P-selectin and E3 to E-selectin), but it was not inhibited by respective mAbs directed at conformational epitopes (P23 to P-selectin and E2 to E-selectin), nor by CSLEX, an IgM antibody to SLe^x. Identical results were also observed using human neutrophils in this cell adhesion assay (data not shown).

DISCUSSION

Selectins are a unique subfamily of Ca^{2+}-dependent animal lectins (22). Structurally, each selectin contains an NH_2-terminal domain of 118 residues homologous to Ca^{2+}-dependent (C-type) animal lectin followed by an epidermal growth factor-like domain, variable numbers of complement regulatory protein-like repeats, a transmembrane domain, and a cytoplasmic tail. Functionally, all selectins mediate Ca^{2+}-dependent cell-cell interactions by binding to sialylated, fucosylated, and, perhaps, sulfated oligosaccharide ligands (a lectin-carbohydrate interaction) (1–5).

Here, we show that only one ~120-kDa glycoprotein from [^{3}H]glucosamine-labeled HL-60 cells can be isolated specifically by E-selectin Rg affinity chromatography in a Ca^{2+}-dependent manner (Figs. 2 and 4). These results suggest that this molecule is an important high affinity glycoprotein ligand for E-selectin. As the molecular mass of the E-selectin ligand thus defined is identical with that of the previously characterized P-selectin ligand (6–10), we would like to know whether they are the same molecules. In this study, we present the following experimental results. (*a*) The ~120-kDa molecule can be cross-depleted by and cross-bound to P- and E-selectin affinity chromatography (Figs. 3 and 5). (*b*) The fingerprints of desialylated *O*-linked carbohydrates from the molecules purified by P- and E-selectin affinity chromatography are identical (Fig. 6). (*c*) The ~120-kDa molecule isolated by sequential affinity chromatographies using PL5 antibody and P-selectin can be recognized by E-selectin Rg and PSGL-1 antiserum (Fig. 8). These results indicate that both E- and P-selectin recognize the same ligand on the HL-60 cells, and this ligand molecule is immunologically identical with PSGL-1, a molecule which has been cloned as a P-selectin glycoprotein ligand by expression cloning (9).

To explore the biological importance of this molecule as a human leukocyte ligand for E-selectin, PL5 mAb, which specifically recognizes the P-selectin glycoprotein ligand from human leukocytes and HL-60 cells and blocks adhesion of these cells to P-selectin (10), was used as a specific inhibitor for cell adhesion to E-selectin. Indeed, PL5 mAb also neutralizes adhesion of HL-60 cells and human neutrophils to E-selectin Rg (Fig. 9). Taken together, our results are fully consistent with previous publications (8, 9) and suggest that the ~120-kDa molecule is an important human leukocyte ligand for both P- and E-selectin. To emphasize the functional importance of this molecule as a ligand for both P- and E-selectin and to keep consistency in the nomenclature (6–10), we will refer to it as the P- and E-selectin glycoprotein ligand-1 (P/ESGL-1).

The surface expression of P- and E-selectin molecules is a highly regulated cellular process (1, 5). P-selectin is stored in the α-granules of platelets and Weibel-Palade bodies of endothelial cells (23, 24). Upon stimulation, this molecule is trans-

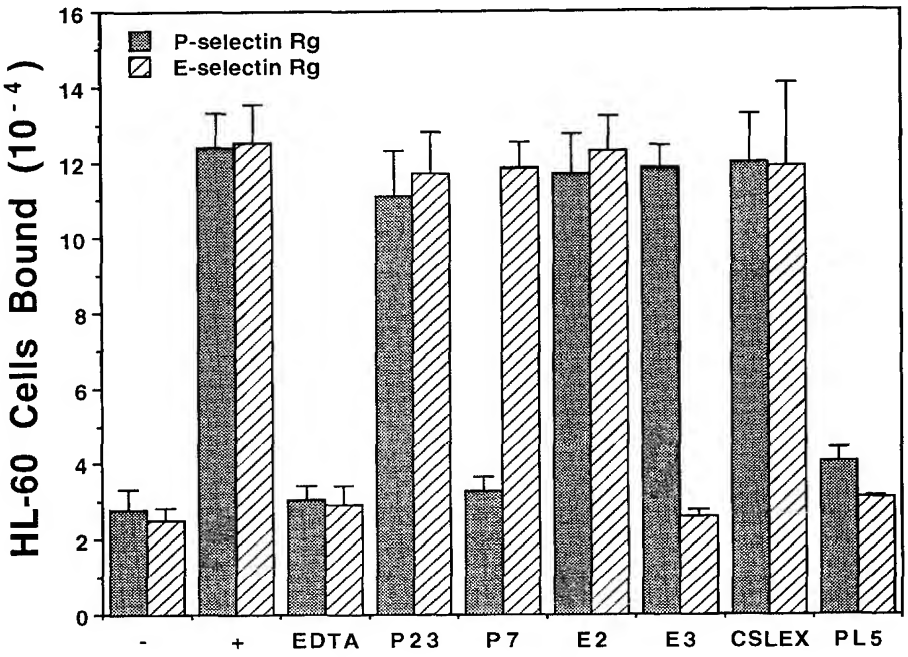

FIG. 9. **PL5 mAb inhibition of adhesion of HL-60 cells to E-selectin Rg.** The cell adhesion assay using P- and E-selectin Rg substrates immobilized on 96-well plates was carried out as described previously (10, 21).[2] HL-60 cells were resuspended in HBSS/fetal calf serum containing 1.26 mM $CaCl_2$ and 0.81 mM $MgCl_2$ except in control wells, designated as *EDTA*, in which they were in HBSS/fetal calf serum containing 2 mM EDTA. HL-60 cells were preincubated with a final concentration of 30 μg/ml PL5 or CSLEX (a mAb to SLe^x). As control for these experiments, P- and E-selectin Rgs were pretreated with a blocking P7 mAb and a nonblocking P23 mAb (both against P-selectin) and with a blocking E3 mAb and a nonblocking E2 mAb (both against E-selectin). Additional control wells immobilized with human IgG were designated as −, and others to which HL-60 cells were added into wells immobilized with selectin substrates without testing antibodies were designated as +. All results were expressed as the mean ± S.D. of adherent cells determined in triplicate measurements of three separate assays.

located to the cell surface of those cells by exocytosis (within minutes) (25, 26). After cell surface expression, P-selectin is rapidly endocytosed back to the cellular granules for storage or, to lysosomes for degradation (27, 28). In contrast, the *de novo* synthesis is required for the cell surface expression of E-selectin exclusively on activated endothelial cells (within hours) (29, 30). The cell surface E-selectin molecules are eventually internalized into lysosomes for degradation (31).

Based on the regulatory features of the cell surface expression of P- and E-selectin molecules, it would appear to us that the cell surface expression of P-selectin is mainly responsible for the early phase (earlier than 2 h) of leukocyte rolling and recruitment at sites of inflammation. In contrast, the cell surface expression of E-selectin is mainly responsible for the later phase (later than 2 h) of leukocyte rolling and recruitment. The compromised leukocyte rolling and recruitment at sites of inflammation in P-selectin-deficient mice predominantly at the early phase (earlier than 2 h) supports this view (32). Therefore, our finding of the P/ESGL-1 molecule as a common leukocyte ligand for both P- and E-selectin suggests that P/ESGL-1 functions as a leukocyte ligand for P-selectin mainly at the early phase of inflammation and for E-selectin mainly at the later phase of inflammation.

Although P/ESGL-1 is expressed constitutively on the cell surface of human leukocytes and HL-60 cells (10), the biosynthesis and regulation of its expression in terms of the biological function in cell adhesion have not been extensively studied as yet. Nagata *et al.* (33) recently reported that granulocyte-like HL-60 cells differentiated by 1.25% Me_2SO released an increased amount of superoxide anion in response to activated platelets in a P-selectin-dependent manner. Whether the differentiated HL-60 cells express more P/ESGL-1 molecules on the cell surface or the same amount of P/ESGL-1 molecules but with a higher affinity for P- and E-selectin remains unknown.

An *O*-linked tetrasaccharide Neuα2–3Galβ1–4(Fucα1–3)GlcNAc, called SLe^x, has been reported as a minimal recognition motif for all three selectins (15, 34–53). As SLe^x is a low affinity ligand for selectins, the multivalent presentation of SLe^x in various forms is required for direct binding to selectins *in vitro* (36, 38–40, 43–45, 51, 52). In this regard, human neutrophil L-selectin has been reported to be a presenter of SLe^x and to function as a ligand for E- and P-selectin (14). Several myeloid cell-derived glycolipids have been shown to be recognized by E-selectin transfected cells in a solid-phase assay. The purified glycolipid contains a sialylated lactosamine with a second internal *N*-acetyllactosamine unit having an α1,3-linked fucose on the *N*-acetylglucosamine (CD65) as analyzed by mass spectroscopy (51). Furthermore, P/ESGL-1 reportedly contains SLe^x, which has been shown to be critical for P-selectin recognition (6–10). The biological importance of the *O*-linked carbohydrates in selectin recognition is further indicated by abolishment of HL-60 cell binding to P-selectin upon treatment of these cells with *Pasteurella haemolytica O*-sialoglycoprotease (EC 3.4.24.57), an enzyme which specifically cleaves proteins that are *O*-glycosylated on clustered serine and threonine residues (7, 33, 36).

In this study, we have released the *O*-linked oligosaccharides from the purified P/ESGL-1 molecules by β-elimination (20). After desialylation to allow better size separation of individual structures, only five peaks of the *O*-linked carbohydrate structures were observed on size exclusion chromatography. Further elucidation of the structures of these *O*-linked oligosaccharides and their structure-function relationships in terms of P- and E-selectin recognition should facilitate the discovery of carbohydrate structure(s) responsible for P- and E-selectin recognition.

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Expression of a P-selectin Ligand in Zona Pellucida of Porcine Oocytes and P-selectin on Acrosomal Membrane of Porcine Sperm Cells. Potential Implications for Their Involvement in Sperm–Egg Interactions

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Abstract. The selectin family of cell adhesion molecules mediates initial leukocyte adhesion to vascular endothelial cells at sites of inflammation. *O*-glycan structural similarities between oligosaccharides from human leukocyte P-selectin glycoprotein ligand-1 (PSGL-1) and from zona pellucida glycoproteins of porcine oocytes indicate the possible existence of a P-selectin ligand in the zona pellucida. Here, using biochemical as well as morphological approaches, we demonstrate that a P-selectin ligand is expressed in the porcine zona pellucida. In addition, a search for a specific receptor for this ligand leads to the identification of P-selectin on the acrosomal membrane of porcine sperm cells. In vitro binding of porcine acrosome-reacted sperm cells to oocytes was found to be Ca^{2+} dependent and inhibitable with either P-selectin, P-selectin receptor–globulin, or leukocyte adhesion blocking antibodies against P-selectin and PSGL-1. Moreover, porcine sperm cells were found to be capable of binding to human promyeloid cell line HL-60. Taken together, our findings implicate a potential role for the oocyte P-selectin ligand and the sperm P-selectin in porcine sperm–egg interactions.

Cell–cell and cell–matrix interactions play central roles in biological development, such as during fertilization, implantation, placenta formation, embryogenesis, cell differentiation, migration, and organ formation. These types of interactions are also essential for a variety of physiological and pathological processes, such as lymphocyte trafficking, immune defense, hemostasis, wound healing, cancer cell invasion, and metastasis. Cell–cell and cell–matrix interactions are precisely controlled and regulated by cell and matrix adhesion molecules with specificities appropriate for their particular functions (Gumbiner, 1992; Hynes and Lander, 1992; Cross et al., 1994; Wassarman, 1995; Snell and White, 1996).

Recruitment of leukocytes, from the flowing blood stream across the endothelial cells of postcapillary venules into the tissue at sites of inflammation or injury, is a multistep paradigm requiring at least three sequential steps. Three major families of cell adhesion molecules are involved in this process: selectins, integrins, and adhesion molecules of the immunoglobulin superfamily (Butcher, 1991; Lasky, 1992; Springer, 1994). The selectins comprise a subfamily of Ca^{2+}-dependent (C-type) animal lectins (Drickamer, 1988, 1993) that are mainly responsible for the initial leukocyte tethering to, and rolling on, the activated endothelial cells. Three members of this family have been described to date (Butcher, 1991; Lasky, 1992; Springer, 1994). L-selectin (CD62L) is a constitutively expressed homing receptor on a majority of leukocytes for lymphatic and vascular endothelial cells. E-selectin (CD62E) is a cytokine-inducible cell surface receptor on vascular endothelial cells, and P-selectin (CD62P) is a rapidly inducible receptor on vascular endothelial cells and platelets. E- and P-selectins function as cell surface receptors for neutrophils, monocytes, T lymphocyte subsets, eosinophils, and basophils.

All three selectins recognize a sialoglycoprotein ligand, P-selectin glycoprotein ligand-1 (PSGL-1)[1] (Moore et al., 1992; Sako et al., 1993; Lenter et al., 1994; Ma et al., 1994; Asa et al., 1995; Spertini et al., 1996; Tu et al., 1996). PSGL-1 is a disulfide-linked dimeric sialomucin expressed on the microvilli of human leukocytes. The recognition of PSGL-1 by the three selectins is Ca^{2+} dependent and sialidase sensitive, characteristics of selectin-mediated leuko-

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1. *Abbreviations used in this paper*: BCECF-AM, 2′,7′-bis(2-carboxyethyl)-5(6)-carboxyfluorescein acetoxylmethyl ester; PGSL-1, P-selectin glycoprotein ligand-1; PUVEC, porcine umbilical vein endothelial cell; Rg, receptor-globulin; TNF-α, tissue necrosis factor-α.

cyte adhesion (Rosen et al., 1985; Yednock and Rosen, 1989; Bevilacqua et al., 1987, 1989; Larsen et al., 1989; Geng et al., 1990).

All three selectins have been reported to bind to the tetrasaccharide structure NeuNAcα2-3Galβ1-4(Fucα1-3) GlcNAc (called sialyl Lewis x or SLe^x) and its isomer, NeuNAcα2-3Galβ1-3(Fucα1-4)GlcNAc (called sialyl Lewis a or SLe^a); these structures may constitute the minimal recognition motif for the three selectins (Brandley et al., 1990; Varki, 1994). In a previous investigation, we isolated PSGL-1 from [^{3}H]glucosamine-labeled HL-60 cells, a human promyeloid cell line, by P- and E-selectin affinity chromatography. The desialylated *O*-linked oligosaccharides released from this molecule were separated into five well-defined peaks having elution volumes corresponding to glucose oligomers composed of 2.5, 3.5, 6.3, 9.8, and 12.8 glucose units, respectively (Asa et al., 1995). Sequencing of the carbohydrate structures in these peaks resulted in the identification of a set of oligosaccharides (Aeed, P., J.-G. Geng, D. Asa, L. Raycroft, L. Ma, and Å. Elhammer, manuscript submitted for publication), which had considerable similarities to structures previously identified on glycoproteins isolated from the porcine zona pellucida (Hirano et al., 1993).

The involvement of oligosaccharide structures in mammalian gamete interactions is well documented (Florman and Wassarman, 1985). In mouse, for instance, *O*-linked oligosaccharides have been specifically implicated in the sperm–egg binding (Wassarman, 1988; Litscher et al., 1995). To determine whether porcine gametes carry cell adhesion molecules similar to those described for the interaction of leukocytes with vascular endothelial cells, i.e., selectins and sialomucin-type ligand molecules (Butcher, 1991; Lasky, 1992; Springer, 1994), we investigated whether a P-selectin ligand and P-selectin are expressed on porcine oocytes and sperm cells, respectively.

Materials and Methods

Materials

Rabbit, mouse, and human IgGs, saponin, L-cysteine, and Hoechst 33258 (H258) were purchased from Sigma Chemical Co. (St. Louis, MO). Mouse IgM was purchased from Calbiochem-Novabiochem Corp. (La Jolla, CA). Calcium ionophore A23187 was purchased from Boehringer Mannheim Biochemicals (Indianapolis, IN). Tissue necrosis factor-α (TNF-α) was purchased from Genzyme (Boston, MA). 2′,7′-bis(2-carboxyethyl)-5(6)-carboxyfluorescein acetoxymethyl ester (BCECF-AM) was purchased from Molecular Probes (Eugene, OR). Paraformaldehyde was purchased from Mallinckrodt Inc. (St. Louis, MO). Glass microscope slides were purchased from Baxter Healthcare Corp. (McGaw Park, IL). Frozen porcine ovaries were obtained from PelFreeze Biologicals (Rogers, AR); fresh porcine ovaries were obtained from a local slaughter house; and fresh porcine umbilical cords were collected from a local farm. The membrane extracts of porcine leukocytes were prepared as previously described (Ma et al., 1994; Asa et al., 1995). HL-60 cells (CCL 240) and Ramos cells (CRL 1596) were obtained from American Tissue Culture Collection (Rockville, MD) and cultured as recommended. All other chemicals were purchased from previously described sources (Ma et al., 1994; Asa et al., 1995).

Proteins and Antibodies

P-selectin was purified from outdated human platelets as published (Ma et al., 1994). E- and P-selectin receptor–globulins (Rg) were prepared as described (Asa et al., 1995).

Monoclonal IgG antibodies against P-selectin, P7 and P23, and monoclonal IgM antibodies against PSGL-1, PL5, and against SLe^x, CSLEX, were prepared and characterized as reported (Ma et al., 1994; Asa et al., 1995). Rabbit polyclonal antibodies raised against human platelet P-selectin and E-selectin Rg were prepared as previously described (Toombs et al., 1995).

A polyclonal IgG antibody against PSGL-1 was prepared as follows. A peptide corresponding to residues 41–55 (QATEYEYLDYDFLPEGGC) of the amino acid sequence of PSGL-1 (Sako et al., 1993) was synthesized on a 430A peptide synthesizer (Applied Biosystems, Inc., Foster City, CA). Two glycine residues (underlined) were added before the carboxyl-terminal cysteine to provide a spacer such that flexibility and availability of the peptide would be retained after conjugation. The peptide was coupled to maleimide-activated keyhole limpet hemocyanin via the carboxyl-terminal cysteine (underlined). Two New Zealand white rabbits (C3633 and C3365; Caltag Laboratories, San Francisco, CA) were immunized with the peptide conjugate. Rabbit IgG fractions were isolated from preimmune sera and antisera using mAb Trap G (Pharmacia Biotech, Piscataway, NJ). The IgG fractions were dialyzed against 27.3 mM Tris-phosphoric acid, pH 6.3, and concentrated on a Mono-Q ion-exchange column (Pharmacia Biotech) by elution with the same buffer containing 2 M NaCl. The purified proteins were then dialyzed against PBS, pH 7.4, and stored at 4°C.

For the immunostaining experiments, the protein G–purified IgG fraction (C3633) was further purified by affinity chromatography on the immobilized antigen peptide as follows. The peptide (2.2 mg) was coupled directly onto ~3 ml of SulfoLink Coupling Gel (Pierce Chemical Co., Rockford, IL); nonspecific binding sites on the gel were blocked with 50 mM L-cysteine, according to the manufacturer's protocol. The IgG fraction was incubated with the immobilized peptide beads at 4°C overnight with end-to-end rotation. After washing with 100 ml of PBS, the bound antibody was eluted with 0.1 M glycine-HCl, pH 2.7. The preparation was then concentrated by Mono-Q chromatography as described above. This procedure yielded ~2 mg of the affinity-isolated antibody protein from ~100 mg of the protein G–purified IgG fraction.

Preparation of Porcine Oocytes and Zona Pellucida

Porcine oocytes and zona pellucida were prepared from frozen or fresh ovaries essentially as described (Dunbar et al., 1980). No obvious contamination by leukocytes could be observed by microscopy. The concentration of total zona pellucida proteins was determined by amino acid compositional analysis.

Isolation of Porcine Sperm Cells

Fresh porcine sperm were collected at a local farm and kept at 37°C until use. No obvious contamination of blood cells could be found in the white milky suspension. The sperm was kept still for at least 15 min at 37°C in the presence of 5% CO_2, and "swim-up" sperm cells were carefully collected on the top layer of the sperm suspension. This was done to avoid the possible contaminating blood cells and to eliminate less viable sperm cells. No obvious contamination by leukocytes could be observed by microscopy.

Preparation of Porcine Umbilical Vein Endothelial Cells

Porcine umbilical vein endothelial cells (PUVEC) were prepared from the freshly collected porcine umbilical cords exactly as previously described for human umbilical vein endothelial cells (Geng et al., 1990; Ma et al., 1994). For induction of E-selectin expression, confluent monolayers of PUVEC (third passage) were treated with 300 U/ml of TNF-α at 37°C for 4 h. After washing once with PBS, the cells were harvested by mechanical detachment with a cell scraper (Nunc, Naperville, IL) in the presence of Versine™ (GIBCO BRL, Gaithersburg, MD).

CHO Cell Line Expressing E-Selectin

A stable CHO-K1 cell line expressing human full-length E-selectin was established by cotransfection of E-selectin cDNA in CDM8 vector (10 μg/ml) with pcDNA1/Neo (1 μg/ml) using a Lipofectin™ (GIBCO BRL) method according to manufacturer's protocol. 3 d later, CHO cells were selected and maintained in DME (high glucose) in the presence of 10% FCS (vol/vol) and 0.4 mg/ml of active Geneticin™ (wt/vol; GIBCO BRL).

SDS-PAGE and Silver Staining

Aliquots of total zona pellucida proteins (~38 μg per lane) and sperm cells (5×10^6 cells per lane; washed three times with ice-cold PBS) were mixed with SDS sample buffer in the presence or absence of 5% β-mercaptoethanol (vol/vol). After boiling for 5 min, samples were subjected to 7% SDS-PAGE. After electrophoresis, proteins were silver stained (Bio Rad Laboratories, Hercules, CA).

Ligand Blotting

Aliquots of total zona pellucida proteins (~115 μg per lane) and porcine leukocyte membrane extracts (~500 μg per lane) were mixed with SDS sample buffer in the presence or absence of 5% β-mercaptoethanol (vol/vol). Samples were boiled for 5 min and subjected to 7% SDS-PAGE. After electrophoresis, proteins were transferred to polyvinylidene difluoride membranes (Immobilon-P; Millipore Corp., Bedford, MA). The membranes were probed with P-selectin followed by biotinylated P23 mAb (1 μg/ml). The membranes were subsequently incubated with a streptavidin–peroxidase complex (Vectostain ABC kit; Vector Laboratories, Burlingame, CA). A chemiluminescent detection system (Amersham Corp., Arlington Heights, IL) was used for detection (Ma et al., 1994; Asa et al., 1995). All incubation and washing buffers contained either 1 mM $CaCl_2$ or 2 mM EDTA as indicated.

Immunoblotting

Aliquots of total zona pellucida proteins (~115 μg per lane), sperm cells (1×10^6 cells per lane), and TNF-α–treated PUVECs (confluent monolayer of cells from a 35-mm dish per lane) were subjected to 7% SDS-PAGE. The separated proteins were transferred to the blotting membranes as described above. For the zona pellucida proteins, the blotting membranes were probed with preimmune IgG or PSGL-1 peptide antibody (both at 1 μg/ml). They were then incubated with biotinylated goat antibodies against rabbit IgG (5 μg/ml). For sperm cells and PUVECs, the blotting membranes were probed separately with either biotinylated rabbit P- or E-selectin antibody or biotinylated P7 mAb (all at 1 μg/ml). The membranes were subsequently incubated with a streptavidin–peroxidase complex followed by a chemiluminescent detection system as described above.

Flow Cytometric Analysis

Porcine sperm cells were used either unwashed or washed three times with HBSS/FCS (1% heat-inactivated FCS in HBSS, vol/vol; 3,000 rpm for 10 min). More than 90% of the washed sperm cells were mechanically capacitated, as determined using Coomassie brilliant blue (Aarons et al., 1991). The sperm cells were resuspended in HBSS/FCS (2×10^6 cells per ml) and incubated separately with either an FITC-conjugated rabbit preimmune IgG, an FITC-conjugated rabbit polyclonal antibody against P- or E-selectin, an FITC-conjugated mouse preimmune IgG, or an FITC-conjugated P7 mAb (all at 10 μg/ml) at 22°C for 1 h. For staining of PUVECs, mechanically detached cells (confluent monolayer of cells from a 35-mm dish per aliquot) were resuspended in HBSS/FCS and incubated with an FITC-conjugated rabbit preimmune IgG or an FITC-conjugated rabbit E-selectin antibody, as outlined above. After incubation, the cells were sedimented by centrifugation at 1,500 rpm for 5 min and washed twice with HBSS/FCS. The cells were then resuspended in HBSS/FCS for flow cytometric analysis (FACScan®; Becton Dickinson & Co., Mountain View, CA).

Immunoelectron Microscopy

Porcine oocytes were washed with HBSS/FCS and incubated with purified platelet P-selectin (10 μg/ml) at 22°C for 1 h. As controls, P-selectin was either omitted or the oocytes were incubated with P-selectin in calcium and magnesium-free HBSS/FCS containing 2 mM EDTA. After washing, the oocytes were incubated with rabbit P-selectin antibody (25 μg/ml) for 30 min. For antibody staining, porcine oocytes were first incubated in 10% (vol/vol) normal goat serum in PBS for 1 h at 22°C. After this step, the oocytes were incubated with 1 μg/ml of PSGL-1 peptide antibody or preimmune IgG for 1 h at 22°C. After washing, samples were incubated with 50 μg/ml of affinity-purified goat anti–rabbit IgG conjugated with HRP (Accurate Chemical Co., Westbury, NY) for 1 h at 22°C. The oocytes were rinsed and fixed with 2.5% glutaraldehyde (vol/vol) and 2% paraformaldehyde (wt/vol) in 0.1 M sodium cacodylate buffer, pH 7.4, containing 0.5 mM $CaCl_2$ at 4°C for 30 min. They were then processed for peroxidase cytochemistry and EM as previously described (Raub et al., 1990).

Rabbit P- and E-selectin antibodies were conjugated with gold particles as previously described (Raub and Kuentzel, 1984). Porcine sperm cells and CHO cells expressing E-selectin were rinsed with HBSS/FCS and incubated with a sixfold dilution of the gold-conjugated rabbit P- or E-selectin antibody in HBSS/FCS at 22°C for 1 h. For inhibition experiments as control, sperm cells were incubated with the gold-conjugated P-selectin antibody in the presence of 200 μg/ml of the unconjugated P-selectin antibody. The labeled cells were fixed with 2% paraformaldehyde (wt/vol) in PBS at 4°C for 15 min followed by 0.1 M ammonium chloride for 5 min. The cells were processed for EM as previously described (Raub and Kuentzel, 1984). Semithick (0.25 μm), en face sections through the cells were viewed with a JEM-1200EX electron microscope (JEOL, Inc., Peabody, MA) operated at 100–120 kV.

Sperm–Egg Binding Assay

A sperm–egg binding assay was set up according to a published procedure (Almeida et al., 1995). Porcine sperm cells and oocytes were gently washed once (1,000 rpm for 10 min) with HBSS/BSA (1% BSA in HBSS; wt/vol) except in controls where calcium and magnesium-free HBSS/BSA containing 2 mM EDTA was used throughout the entire assay. The sperm (0.1-ml aliquots of 2×10^6 cells per ml) were mixed with oocytes (0.1-ml aliquots of ~500 oocytes per ml), in the presence or absence of 5 μM A23187, at 22°C for 1 h. The cell mixtures were carefully layered on the top of 100% dialyzed and heat-inactivated FCS (1 ml per tube) and spun at 500 rpm on a table-top centrifuge for 2 min (FCS "cushion"). Under these washing conditions, <10% of the free sperm cells were mechanically capacitated as determined using Coomassie brilliant blue (Aarons et al., 1991), and more than 90% of the free sperm cells were viable according to their ability to exclude Hoechst 33258 (Tao et al., 1993). The supernatants were discarded and the cell pellets were fixed with 0.2 ml per tube of 2% paraformaldehyde (freshly prepared in PBS, wt/vol). The preparations were transferred onto glass microscope slides and examined under a microscope (Nikon Phase Contrast-2, EL WD 0.3; Tokyo, Japan), equipped with a screen monitor (VOCON Industries, Inc., New York) and a VC2400 video camera (VICON Industries, Inc., New York). The microscopic images were printed using a color video printer (UP-5200MD; Sony, Park Ridge, NJ).

For the inhibition studies, sperm cells were preincubated with mouse IgG, P23 mAb, or P7 mAb (all at 30 μg/ml) in the presence of 5 μM A23187 at 22°C for 30 min. Oocytes were preincubated with mouse IgM, CSLEX mAb, PL5 mAb, or human IgG, E-selectin Rg, or P-selectin Rg (all at 30 μg/ml) at 22°C for 30 min. The cells were then mixed with sperm cells without washing for the binding assay, as described above.

Sperm Cell–HL-60 Cell Binding Assay

Freshly collected sperm (0.3 ml) were resuspended into 30 ml of HBSS/BSA and loaded with 2 μM BCECF-AM at 37°C for 30 min (Ma et al., 1994; Asa et al., 1995). The labeled sperm cells as well as HL-60 and Ramos cells were washed three times with PBS (1,000 rpm for 10 min). The cells were resuspended in HBSS/BSA (2×10^6 cells per ml), except in controls where calcium and magnesium-free HBSS/BSA containing 2 mM EDTA was used, throughout the assay. Under these washing conditions, ~30–50% of the sperm cells were mechanically capacitated as determined using Coomassie brilliant blue (Aarons et al., 1991), and >80% of the free sperm cells were viable based on their ability to exclude Hoechst 33258 (Tao et al., 1993).

The labeled sperm cells (1×10^6 cells in a 0.5-ml aliquot) were mixed with either Ramos cells or HL-60 cells (2×10^5 cells in a 0.1-ml aliquot) in the presence or absence of 5 μM A23187 at 22°C for 1 h, with end-to-end rotating. The unbound sperm cells were removed on a FCS cushion (0.5 ml of FCS per tube, centrifuged at 700 rpm on a table-top centrifuge for 2 min). The cell pellets were fixed with 2% paraformaldehyde (wt/vol; 0.5 ml per tube) for flow cytometric analysis (FACScan®). The binding of the fluorescence-labeled sperm cells to the Ramos or HL-60 cells was measured as the mean fluorescence intensity from >100,000 cells in the gated windows for Ramos or HL-60 cells.

For the inhibition studies, the labeled sperm cells were preincubated with mouse IgG, P23 mAb, or P7 mAb (all at 30 μg/ml) in the presence of 5 μM A23187 at 22°C for 30 min. HL-60 cells were preincubated with either mouse IgM, CSLEX mAb, PL5 mAb, or human IgG, E-selectin Rg, or P-selectin Rg (all at 30 μg/ml) at 22°C for 30 min. The cells were subsequently mixed with oocytes without washing for the binding assay.

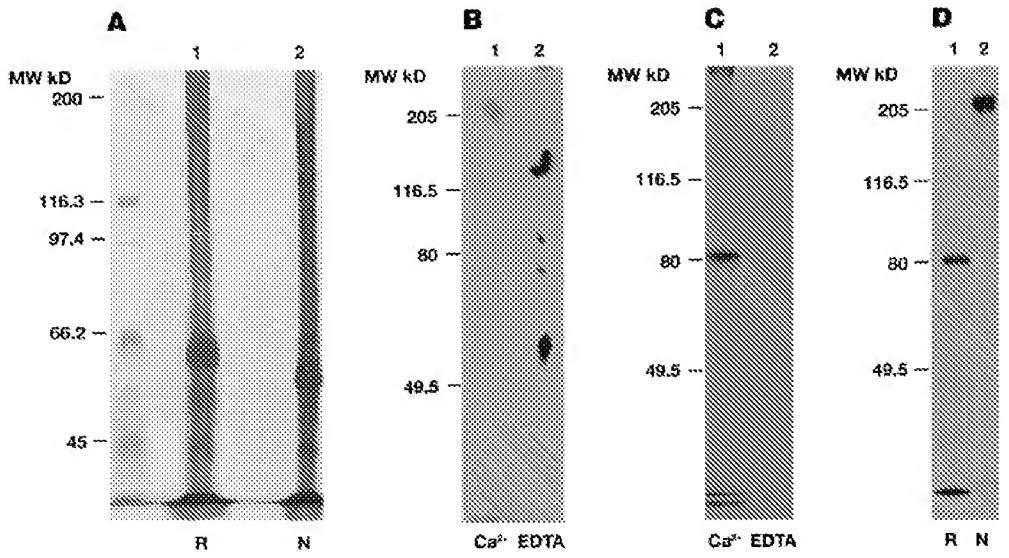

Figure 1. Ca^{2+}-dependent binding of P-selectin to a dimeric molecule from porcine zona pellucida. (*A*) Silver staining of total zona pellucida proteins (~38 μg per lane) separated on 7% SDS-PAGE in the presence (*R*) or absence (*N*) of 5% β-mercaptoethanol (vol/vol). (*B*) P-selectin ligand blotting of zona pellucida proteins (~115 μg per lane), fractionated on 7% SDS-PAGE under nonreducing conditions. After electrophoresis, the proteins were transferred onto a blotting membrane and probed with human platelet P-selectin followed by a biotinylated P23 mAb in the presence of 1 mM $CaCl_2$ or 2 mM EDTA, as indicated. The blots were visualized with a chemiluminescence detection system (Amersham Corp.). (*C*) P-selectin ligand blotting of the zona pellucida proteins under reducing conditions. (*D*) P-selectin ligand blotting of the membrane extracts of porcine leukocytes (~0.5 mg per lane) under reducing (*R*) and nonreducing (*N*) conditions. Blotting procedures were exactly as described for *B*.

Results

Expression of a P-selectin Ligand in Zona Pellucida of Porcine Oocytes

To investigate whether the zona pellucida of porcine oocytes contains a specific ligand for P-selectin, we took advantage of the cross-reactivity of human platelet P-selectin with porcine neutrophils, i.e., the fact that human P-selectin supports adhesion of porcine neutrophils (Geng, J.-G., unpublished observations). As shown in Fig. 1 *A*, porcine zona pellucida contains many proteins as visualized by silver staining. Based on densitometric measurements, >50%

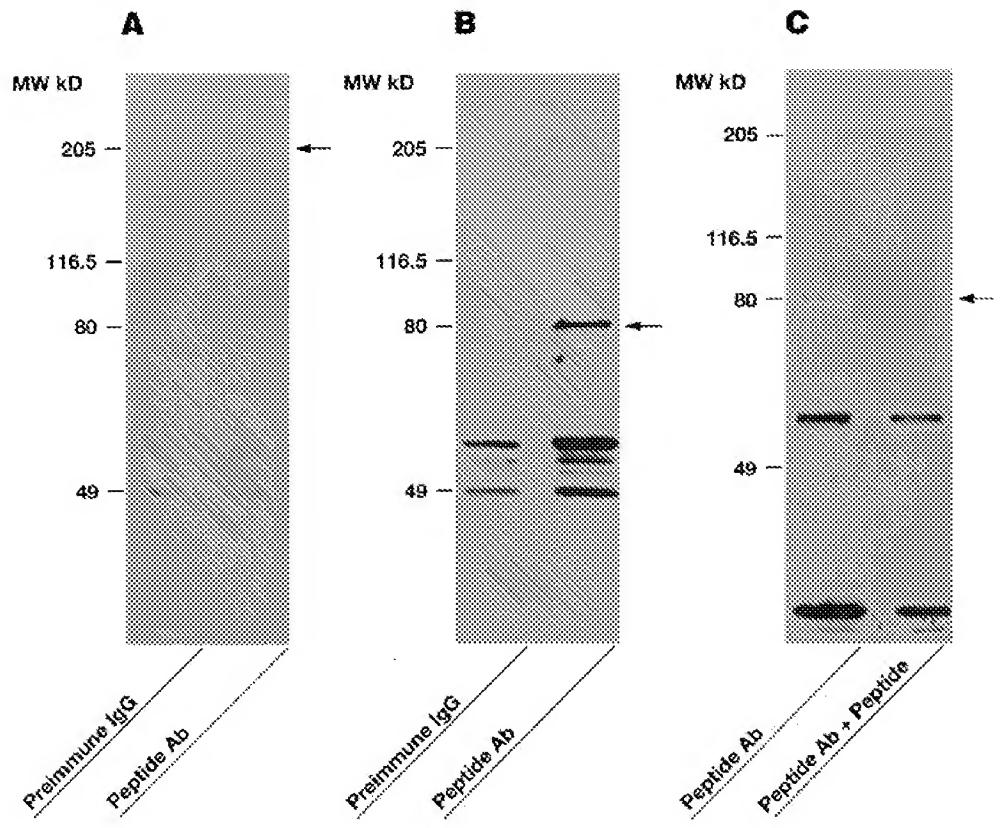

Figure 2. Immunoblotting of zona pellucida proteins with PSGL-1 peptide antibody. Porcine zona pellucida proteins, fractionated by 7% SDS-PAGE under nonreducing (*A*) and reducing (*B* and *C*) conditions and transferred to blotting membranes, were probed with rabbit preimmune IgG or PSGL-1 peptide antibody followed by biotinylated antibody to rabbit IgG. Immunoreactive proteins were visualized as outlined in the legend to Fig. 1.

of the proteins were recovered in the 40–70-kD ranges, under reducing conditions. This is consistent with published data (Dunbar et al., 1980).

In a parallel experiment, the zona pellucida proteins, separated by 7% SDS-PAGE and transferred to blotting membranes, were probed with human platelet P-selectin. Surprisingly, P-selectin specifically bound to a single protein with a molecular mass of ~210 kD under nonreducing conditions (Fig. 1 *B*, lane *1*) and ~80 kD under reducing conditions (Fig. 1 *C*, lane *1*). The binding was Ca^{2+} dependent as it was completely abolished by performing the P-selectin incubation in the presence of 2 mM EDTA (Fig. 1, *B* and *C*, lanes *2*). P-selectin also specifically recognized a dimeric molecule from porcine leukocyte membrane extracts, with molecular masses identical to those of the zona pellucida protein under both nonreducing and reducing conditions (Fig. 1 *D*).

Three additional weaker bands (at ~210, ~180, and ~140 kD under reducing conditions) were also recognized by P-selectin (Fig. 1 *C*, lane *1*). The presence of the ~210-kD band may be due to incomplete reduction of the ~210-kD dimer, a phenomenon frequently observed for human leukocyte PSGL-1 (Moore et al., 1992; Sako et al., 1993; Lenter et al., 1994). The nature of ~180- and ~140-kD bands is unclear; they were not recognized by P-selectin (Fig. 1 *B*, lane *1*) and PSGL-1 peptide antibody (see Fig. 3 *A*) when proteins were separated under nonreducing conditions. Plus, they were not recognized by PSGL-1 peptide antibody (Fig. 3, *B* and *C*), under reducing conditions. In addition, there was some background staining spots on the blots, which were not similar to the protein bands (Fig. 1 *B*, lane *2*).

To corroborate the above findings, the porcine zona pellucida proteins were also probed with an antibody against a synthetic peptide encoding residues 41–55 of the amino acid sequence of PSGL-1. Fig. 2 shows that this antibody, but not preimmune IgG, bound to the ~210-kD protein under nonreducing conditions (*A*, *arrow*) and the ~80-kD protein under reducing conditions (*B*, *arrow*). Preincubation of the antibody with the synthetic peptide abrogated this binding (*C*, *arrow*). The protein bands at ~50–60 kD were most likely due to nonspecific binding, since (*a*) they existed in the blots probed with both preimmune IgG and PSGL-1 peptide antibody (*A* and *B*), and (*b*) they were not inhibited with the respective peptide antigen (*C*).

Using EM, the distribution of the P-selectin ligand in the zona pellucida of porcine oocytes was examined after labeling either with P-selectin followed by P-selectin antibody or with PSGL-1 peptide antibody. The experiments demonstrated that the P-selectin ligand was associated with membrane fragments and vesicles embedded throughout

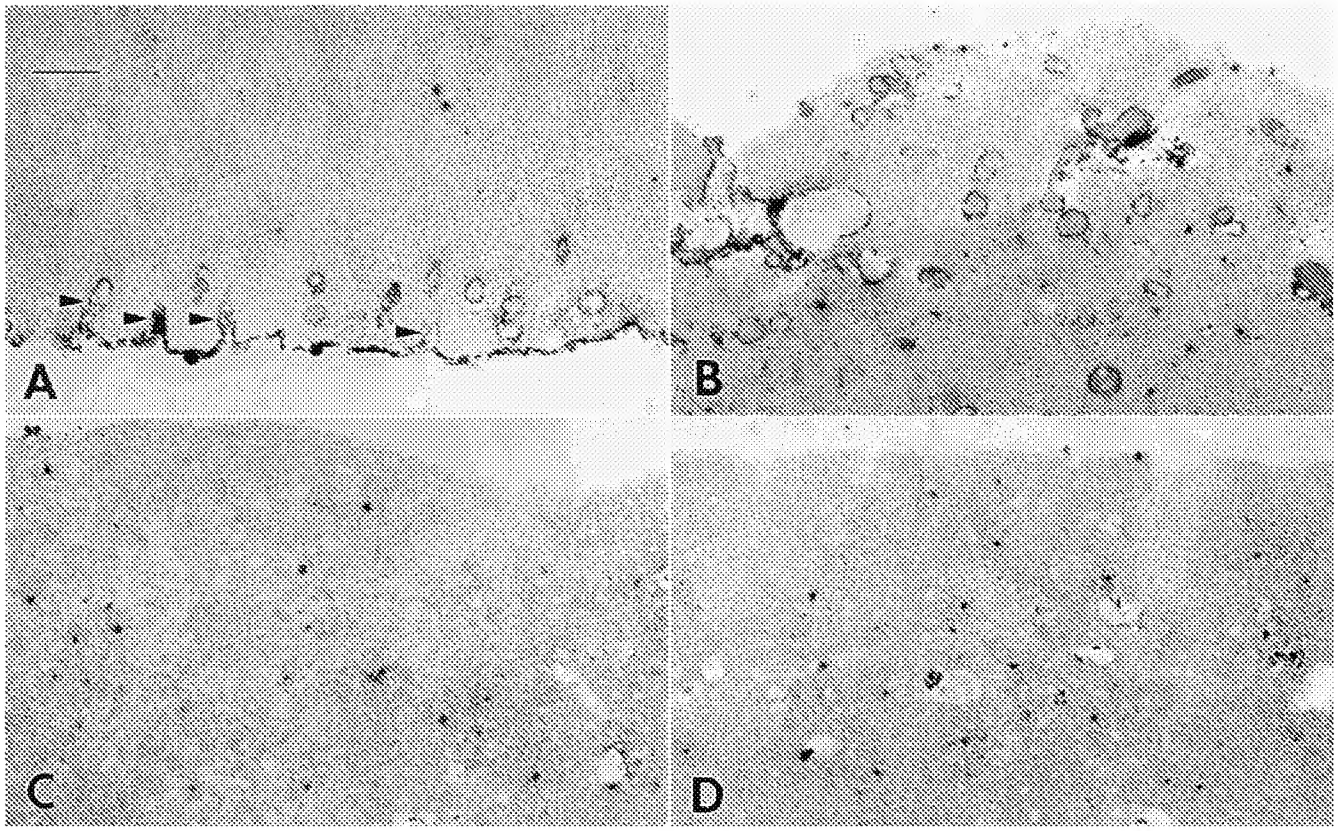

Figure 3. Distribution of a P-selectin ligand in porcine zona pellucida. Porcine oocytes were washed and incubated sequentially with platelet P-selectin, rabbit P-selectin antibody, and an HRP-conjugated secondary antibody. P-selectin binding was visualized by the electron-dense reaction product. P-selectin is bound to the membrane vesicles and fragments embedded within the zona pellucida at or near the (*A*) inner surface (*arrows*) of the zona pellucida, which is adjacent to the oocyte removed during the staining procedures, and (*B*) outer surface. (*C*) Zona pellucida stained in the absence of P-selectin. (*D*) Zona pellucida stained with P-selectin in the presence of EDTA. Bar, 500 nm.

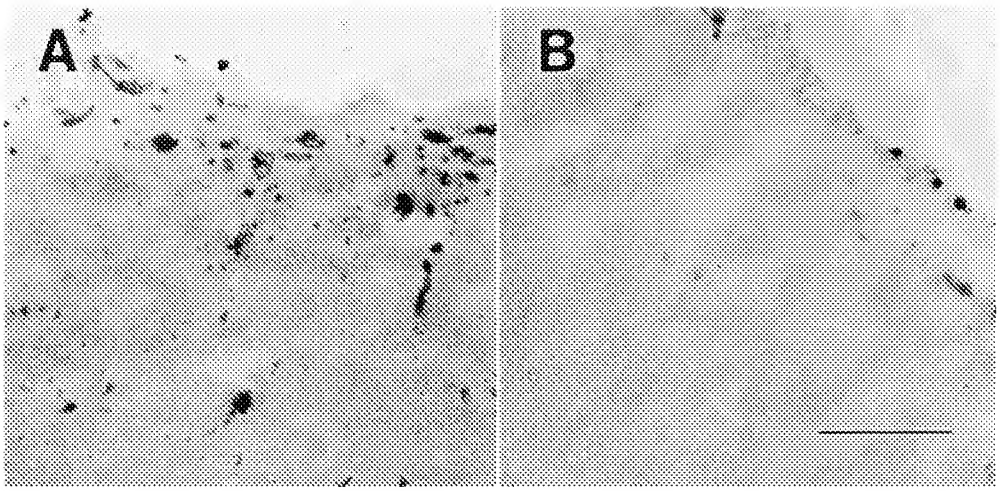

Figure 4. Staining of porcine zona pellucida with PSGL-1 peptide antibody. Zona pellucida of porcine oocytes were stained with either (*A*) PSGL-1 peptide antibody or (*B*) preimmune IgG followed by indirect immunoperoxidase EM as described above. The outer surfaces of the zona are shown. Bar, 2 μm.

the matrix of the zona pellucida (Figs. 3 and 4). The specificities of these approaches were confirmed by the absence, or marked reduction, of peroxidase reaction product in control oocytes, where either P-selectin (Fig. 3 *C*) or P-selectin antibody (data not shown) was omitted, where P-selectin was incubated in the presence of 2 mM EDTA (Fig. 3 *D*), or where preimmune IgG was used (Fig. 4 *B*).

P-selectin Expression on Acrosomal Membrane of Porcine Sperm Cells

The expression of P-selectin on porcine sperm cells was first established by FACS® analysis, using two different P-selectin antibodies, an FITC-conjugated rabbit P-selectin antibody and an FITC-conjugated P7 mAb. Both antibodies were raised against human platelet P-selectin and both reacted with porcine platelet P-selectin (see Fig. 7 *C*). As shown in Fig. 5, rabbit P-selectin antibody (*A*) and P7 mAb (*B*) bound to repeatedly washed sperm cells whose plasma membranes were no longer intact (see Fig. 8). By contrast, FITC-conjugated rabbit E-selectin antibody did not bind to the sperm cells (Fig. 5 *C*), although it clearly reacted with the TNF-α–treated PUVEC (*D*). Interestingly, P-selectin polyclonal antibody did not bind to the unwashed sperm cells (Fig. 6 *A*) unless they were treated with A23187 (a calcium ionophore known to induce the acrosomal reaction; Fig. 6 *B*), repeated washing (causing disruption of the plasma membranes, as demonstrated in Fig. 8; Fig. 6 *C*), or saponin (a detergent that selectively permeabilizes the plasma membrane; Fig. 6 *D*). Together, these results suggest that porcine sperm cells express P-selectin, but not E-selectin, and that P-selectin is expressed on the acrosomal membrane of the sperm cells, but not on the plasma membrane.

To corroborate the finding of P-selectin expression on porcine sperm cells, an immunoblotting experiment was carried out. Intact sperm cells were washed with ice-cold PBS and lysed in SDS sample buffer. The total sperm cell proteins were separated by SDS-PAGE and stained by silver staining. This resulted in numerous protein bands with various molecular masses, under reducing and nonreducing conditions (Fig. 7 *A*). The separated proteins were also transferred to blotting membranes and probed with either rabbit P- or E-selectin antibody or with P7 mAb. Both P-selectin antibodies bound to ~120-kD proteins under nonreducing conditions (Fig. 7 *B*, *arrow*). The observed molecular mass is identical to the molecular mass for human platelet P-selectin (Ma et al., 1994) and porcine platelet P-selectin (Fig. 7 *C*) (Toombs et al., 1995). The identities of the additional protein bands, with lower molecular masses, observed on the blots are not known. They may represent proteolytic fragments of P-selectin generated

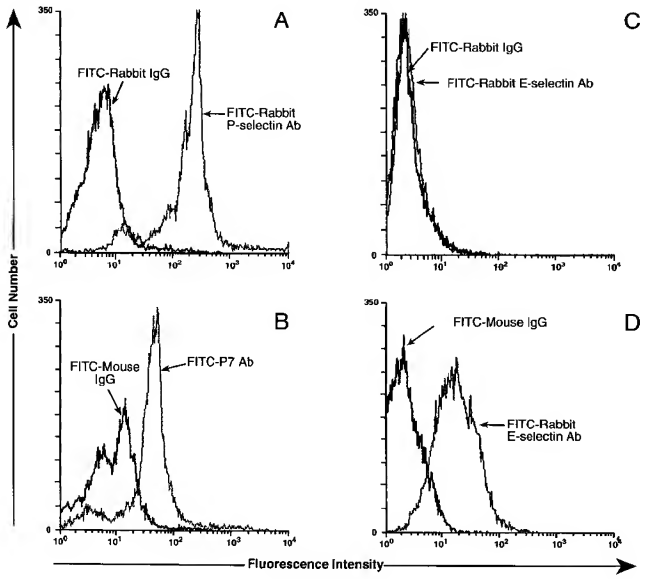

Figure 5. P-selectin antibody binding to sperm cells. Repeatedly washed porcine sperm cells (2×10^6 cells per ml) were incubated separately with FITC-labeled rabbit IgG, an FITC-labeled rabbit P-selectin antibody (*A*), FITC-labeled mouse IgG, an FITC-labeled P7 mAb against P-selectin (*B*), or an FITC-labeled rabbit E-selectin antibody (*C*), respectively, at 22°C for 1 h (all at 10 μg/ml). Similarly, TNF-α–treated PUVECs were incubated separately with FITC-labeled rabbit IgG or an FITC-labeled rabbit E-selectin antibody (*D*). After washing, the sperm and endothelial cells were analyzed by flow cytometry (FACScan®). Results were presented as histograms of log fluorescence intensities over cell numbers from 10,000 cells.

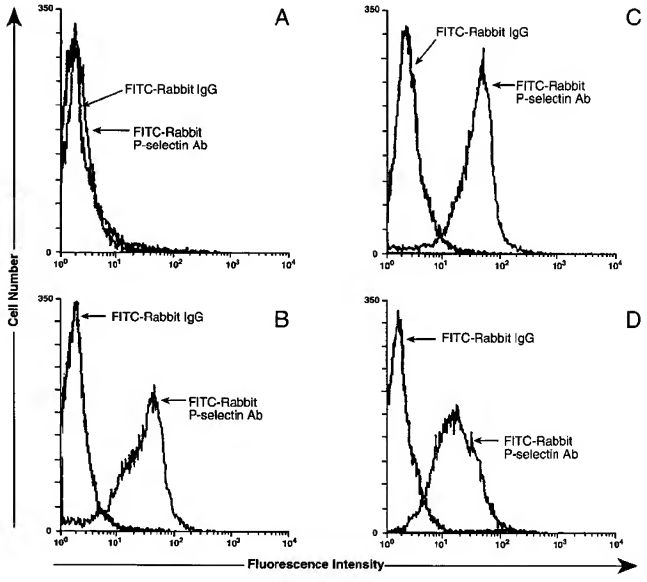

Figure 6. P-selectin antibody binding to sperm cells after acrosomal reaction or permeablization. Porcine sperm cells (2×10^6 cells per ml) without prior washing (*A*), without prior washing in the presence of 5 μM A23187 (*B*), with prior repeated washing (*C*), and without prior washing in the presence of 0.01% saponin (*D*) were incubated with either FITC-labeled rabbit IgG or FITC-labeled rabbit P-selectin antibody at 22°C for 1 h (all at 10 μg/ml). After washing, the cells were analyzed by flow cytometry as above.

during the lysis of the sperm cells, since (*a*) sperm cells were known to contain a variety of proteases (Eddy, 1988), and (*b*) the antibodies used in this experiment specifically bound to P-selectin among all the proteins present in the platelet lysates (Fig. 7 *C*). The relatively sharper ~120-kD bands from sperm cells (Fig. 7 *B*, *arrow*), as compared with the relatively broad bands from platelets (Fig. 7 *C*), could also result from the proteolytic cleavage of some forms of the sperm P-selectin. Differential protease accessibility of heterogeneous glycosylated platelet P-selectin has been demonstrated previously (Johnston et al., 1989). E-selectin antibody did not recognize any protein in the porcine sperm cells, although it bound avidly to a ~90-kD protein from TNF-α–treated PUVEC (Fig. 7 *D*).

Further studies, using immunoelectron microscopy and a gold-conjugated rabbit P-selectin antibody, revealed that the localization of P-selectin on the porcine sperm cells was confined to the region of the sperm head, containing the dense nucleus, covered only by the exposed acrosome (Fig. 8 *A*, *1*), including the acrosomal cap (Fig. 8 *A*, *arrow*). The gold-conjugated P-selectin antibody did not label the lower one-third of the sperm head (Fig. 8 *A*, *2*), the neck (Fig. 8 *A*, *3*), or the tail (Fig. 8 *A*, *4*). Consistent with the results from the flow cytometric studies (Fig. 6), label was not observed on sperm cells with the plasma membrane still intact (Fig. 8 *B*). Incubation of sperm cells with the gold-conjugated P-selectin antibody in the presence of the unconjugated P-selectin antibody abolished the binding (Fig. 8 *C*). Again, gold-conjugated rabbit E-selectin antibody did not label the sperm cells (Fig. 8 *D*). Neither the broken plasma membrane (Fig. 8 *D*, *arrow*) nor the acrosome was labeled, although it labeled the surface of a CHO cell line expressing human E-selectin (Fig. 8 *E*). Thus, these results confirm the expression of P-selectin, but not E-selectin, on the acrosomal membranes, but not on the plasma membrane, of porcine sperm cells. Furthermore, the detection of P-selectin after acrosomal reaction suggests the possible localization of the molecule on the inner acrosomal membrane.

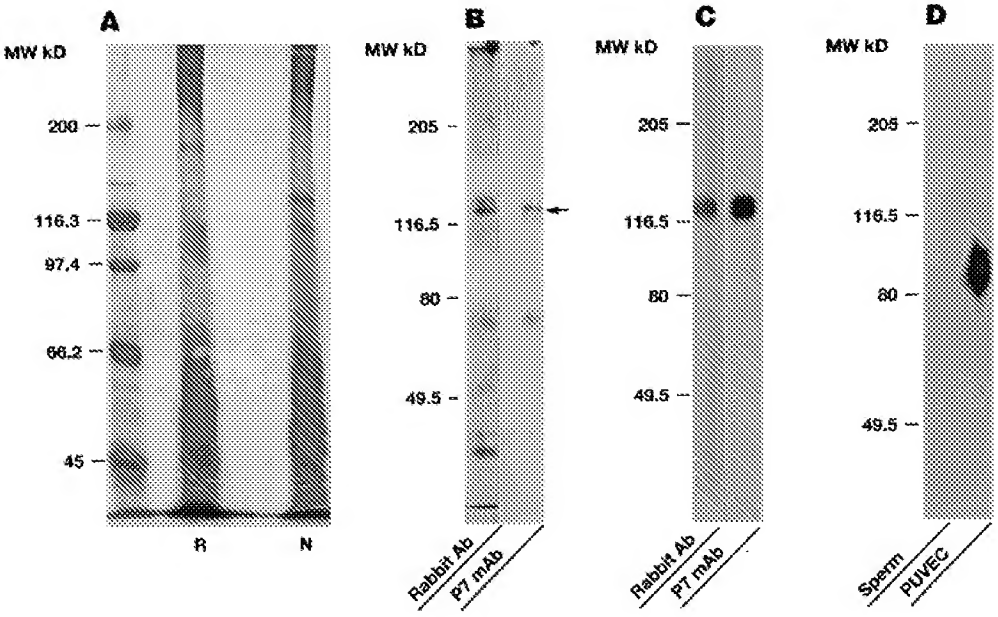

Figure 7. Immunoblotting of porcine sperm cells. Porcine sperm cells (5×10^5 cells per lane for silver staining and 1×10^6 cells per lane for immunoblotting), porcine platelets (1×10^5 cells per lane), and PUVEC (confluent monolayer of cells from a 35-mm dish per lane) were mixed with SDS sample buffer and boiled for 5 min. After electrophoresis under reducing (*A*, *R*) and nonreducing conditions (*A*, *N*; *B–D*), proteins were either silver stained (*A*) or transferred to blotting membranes and probed with 1 μg/ml of biotinylated rabbit P-selectin antibody, biotinylated P7 mAb against P-selectin (*B* for sperm cells and *C* for platelets), or biotinylated rabbit E-selectin antibody (*D*). Immunoreactive proteins were visualized as outlined in the legend to Fig. 1.

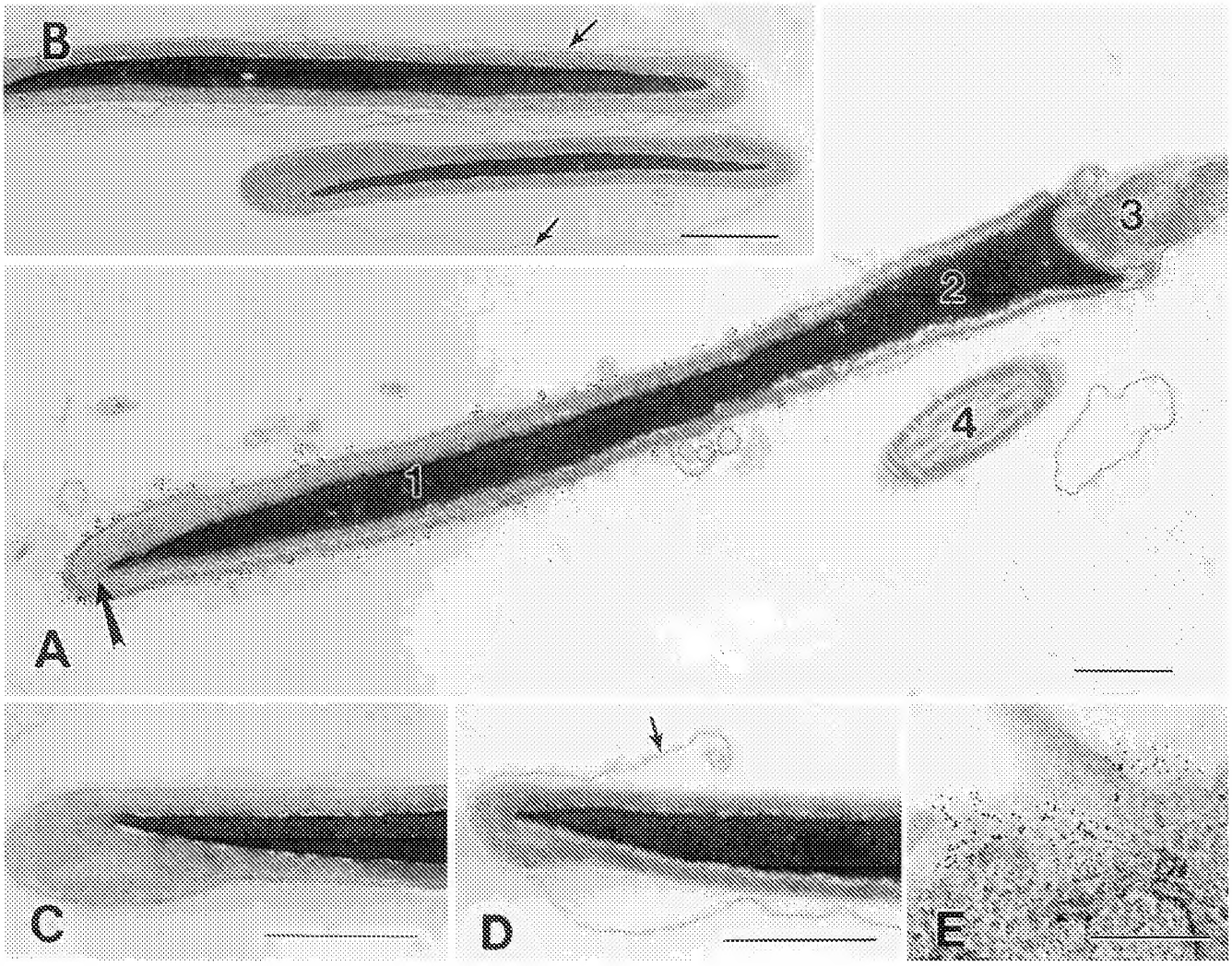

Figure 8. Localization of P-selectin on sperm cells. Washed sperm cells were incubated with a gold-conjugated P- or E-selectin antibody and examined by EM. (*A*) The staining of P-selectin antibody was confined to the region of the sperm head (*1*), which contained the dense nucleus, covered only by the acrosome including the acrosomal cap (*arrow*). P-selectin was not expressed on the lower third of the head region (*2*), the neck (*3*), or the tail (*4*). (*B*) The gold-conjugated P-selectin antibody did not label sperm cells with the plasma membrane (*arrow*) still intact. (*C*) Control sperm cell was incubated with the gold-conjugated P-selectin antibody in the presence of 200 μg/ml of the unconjugated P-selectin antibody. (*D*) The gold-conjugated E-selectin antibody did not label the sperm cell. Neither the broken plasma membrane (*arrow*) nor the acrosome were labeled. (*E*) The gold-conjugated E-selectin antibody labeled the cell surface of a CHO cell expressing human E-selectin. Bar, 0.5 μm.

Function of the Oocyte P-selectin Ligand and the Sperm Cell P-selectin

To evaluate the function of the P-selectin ligand in the zona pellucida of porcine oocytes and P-selectin on the acrosomal membrane of porcine sperm cells, an in vitro sperm–oocyte binding assay was carried out, essentially according to the published procedure (Almeida et al., 1995). In this assay, acrosome-reacted sperm cells bound to oocytes in numbers usually exceeding 15 sperm cells per oocyte (referring to the number of bound sperm cells viewed in the single optical plane used; the total number of sperm cells bound to the entire oocyte was considerably larger; Table I). The binding was Ca^{2+} and Mg^{2+} dependent, since it could be dramatically reduced (to less than three sperm cells per oocyte) by treatment with EDTA. The requirement for divalent cations for sperm–egg binding is consistent with previous observations (Yanagimachi, 1988).

Sperm–egg binding was also significantly reduced (to less than five sperm cells per oocyte) by preincubation of the sperm cells with P7 (a leukocyte adhesion blocking mAb against P-selectin), or by preincubation of the oocytes with PL5 (a leukocyte adhesion blocking mAb against PSGL-1), purified platelet P-selectin, or recombinant P-selectin Rg (Table I). By contrast, the sperm–egg binding was not affected by preincubation of the oocytes with mouse IgM, CSLEX (an mAb against SLe^x), mouse IgG, P23 (a leukocyte adhesion nonblocking mAb against P-selectin), or E-selectin Rg (Table I).

These results strongly suggest that both the P-selectin ligand in the zona pellucida of porcine oocytes and P-selectin on the acrosome-reacted sperm cells are biologically func-

Table I. Adhesion of Sperm Cells to Oocytes

Condition	Sperm cells per oocyte*
–	18 ± 3
EDTA	1 ± 1
Mouse IgG	20 ± 4
P23 mAb	19 ± 5
P7 mAb	4 ± 3
Mouse IgM	19 ± 6
CSLEX mAb	17 ± 2
PL5 mAb	5 ± 4
Human IgG	17 ± 2
P-selectin Rg	2 ± 2
E-selectin Rg	16 ± 5
P-selectin	3 ± 1

Porcine sperm cells (2×10^6 cells per ml) and oocytes (~500 oocytes per ml) were resuspended in HBSS/BSA (–), or calcium and magnesium–free HBSS containing 2 mM EDTA (*EDTA*). The sperm cells and oocytes were mixed in the presence of A23187 unless specifically indicated. Aliquots of oocytes were preincubated separately with mouse IgM, CSLEX (an mAb against SLe^x), PL5 (an mAb against PSGL-1; all at 30 μg/ml), or platelet P-selectin (10 μg/ml), or human IgG, P-, and E-selectin Rgs (all at 30 μg/ml), respectively. Aliquots of sperm cells were preincubated with mouse IgG, P7 (a leukocyte adhesion blocking mAb against P-selectin), or P23 (a leukocyte adhesion nonblocking mAb against P-selectin; all at 30 μg/ml) at 22°C for 30 min. All results were expressed as the mean ± SD number of adherent sperm cells per oocyte in the optical plane studied in five separate experiments; more than 50 oocytes were observed for each condition.

*Refers to the number of bound sperm cells viewed in the single optical plane used.

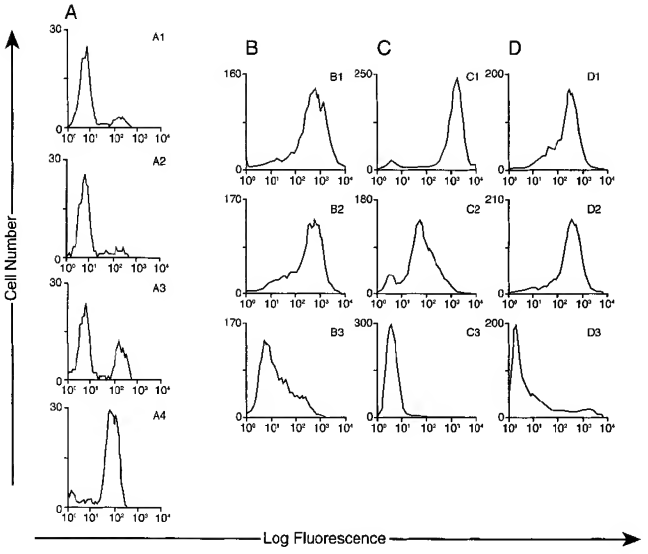

Figure 9. Binding of porcine sperm cells to human myeloid cell line HL-60. Freshly collected sperm cells were loaded with a fluorescent dye, BCECF-AM. The labeled sperm cells (1×10^6 cells in 0.5-ml aliquots) were mixed with either Ramos cells or HL-60 cells (2×10^5 cells in 0.1-ml aliquots) at 22°C for 1 h in the presence of 5 μM A23187, 1.26 mM $CaCl_2$, and 0.81 mM $MgCl_2$, unless otherwise indicated. After removal of unbound sperm cells, the cell pellets were fixed with 2% paraformaldehyde. Binding of the fluorescence-labeled sperm cells to Ramos or to HL-60 cells was measured by flow cytometric analysis (FACScan®). (*A*) Binding of labeled sperm cells to Ramos cells in the presence of EDTA (*A1*) or A23187 (*A2*). Binding of labeled sperm cells to HL-60 cells in the absence (*A3*) or presence (*A4*) of A23187. (*B*) Binding of labeled sperm cells to HL-60 cells in the presence of mouse IgG (*B1*), P23 mAb (a leukocyte adhesion nonblocking mAb against P-selectin; *B2*), or P7 mAb (a leukocyte adhesion blocking mAb against P-selectin; *B3*). (*C*) Binding of labeled sperm cells to HL-60 cells in the presence of mouse IgM (*C1*), CSLEX (an mAb against SLe^x; *C2*), or PL5 (an mAb against PSGL-1; *C3*). (*D*) Binding of labeled sperm cells to HL-60 cells in the presence of human IgG (*D1*), E-selectin Rg (*D2*), or P-selectin Rg (*D3*).

tional. To corroborate this finding, we investigated whether porcine sperm cells could bind to HL-60 cells, a human promyeloid cell line that expresses the functional PSGL-1 (Moore et al., 1992; Sako et al., 1993; Lenter et al., 1994; Ma et al., 1994; Asa et al., 1995; Spertini et al., 1996; Tu et al., 1996). Fig. 9 shows that, in the presence of Ca^{2+}, Mg^{2+}, and A23187, fluorescently labeled sperm cells avidly bound to HL-60 cells (A4), but not to Ramos cells (A1 and A2), a human lymphoblast cell line that does not express the functional PSGL-1 (Vachino et al., 1995). This binding was reduced when the experiment was carried out in the absence of A23187 (A3), a calcium ionophore that induces the sperm acrosomal reaction (Aarons et al., 1991; Tao et al., 1993). The partial binding observed in the absence of A23187 (A3) was likely attributed to the broken cytoplasmic membrane on some sperm cells, caused by the repeated washing procedures used in their preparation (Fig. 8). The requirement for A23187 for full binding activity is consistent with the expression of P-selectin on the acrosomal membrane of sperm cells (Figs. 6 and 8).

As expected, the binding activity was blocked by preincubation of the sperm cells with P7 (a leukocyte adhesion blocking mAb against P-selectin; B3), but not with mouse IgG (B1) or P23 (a leukocyte adhesion nonblocking mAb against P-selectin; B2). Preincubation of HL-60 cells with PL5 (a leukocyte adhesion blocking mAb against PSGL-1; C3) or P-selectin Rg (D3) also neutralized the binding, but mouse IgM (C1), human IgG (D1), or E-selectin Rg (D2) did not. Taken together, the results provide independent and convergent evidence for the biological function of the zona pellucida P-selectin ligand and the sperm P-selectin.

It should be mentioned that the anti-SLe^x mAb, CSLEX, partially inhibited the interaction in this assay. This partial inhibition, by CSLEX mAb, of the binding of sperm cells to HL-60 cells (Fig. 9; *C2*), but not on the binding of sperm cells to the oocytes (Table I), may be due to subtle differences in assay formats. Alternatively, the difference in cell type (different oligosaccharide structures on porcine oocytes vs human HL-60 cells) and/or the particulars of the CSLEX mAb specificity (recognizes other carbohydrate epitopes besides SLe^x; Stroud et al., 1996*a*,*b*) may contribute to this partial inhibition. In addition, the lack of inhibition by E-selectin Rg, particularly in sperm cell binding to HL-60 cells (Fig. 9; *D2*), raises several possibilities, such as (*a*) there is an insufficient amount of E-selectin Rg in the assay; (*b*) the binding determinant(s) on PSGL-1 for P- and E-selectin is quite distinct and separated; (*c*) there is sufficient other ligand(s) for E-selectin; or (*d*) PSGL-1 is not a functional ligand for E-selectin. Obviously, further experiments are required to clarify these issues.

Discussion

Attachment of the sperm cell to the oocyte is the first step in mammalian fertilization. This process involves a cascade of cell–cell and cell–matrix interactions with at least

six consecutive steps (Wassarman, 1995; Snell and White, 1996). Sperm cells first bind to the zona pellucida, a large extracellular matrix surrounding the oocyte. This binding triggers the acrosomal reaction to facilitate the penetration of the sperm cell through the zona pellucida. After penetration, the sperm cell binds to and fuses with the plasma membrane of the oocyte. The fertilized egg finally undergoes implantation, placenta formation, and embryonic development.

Several adhesion molecules have been implicated to play important roles in gamete binding. The sperm binding to the zona pellucida in mouse is reportedly mediated by ZP3 glycoprotein (Bleil and Wassarman, 1980), a reaction that induces the acrosomal reaction on the mouse sperm cells (Bleil and Wassarman, 1983). Interestingly, this interaction appears to be mediated by *O*-linked carbohydrates on ZP3 molecule (Florman and Wassarman, 1985; Litscher et al., 1995). On the other hand, the binding of sperm cells to the plasma membrane of the oocyte is mediated by fertilin, a mouse sperm surface protein, and its counterligand, $\alpha 6\beta 1$ integrin, on the oocyte surface (Blobel et al., 1992; Almeida et al., 1995).

The specific distribution pattern of the P-selectin ligand in the zona pellucida matrix argues against the possibility that the detection of the ligand in this tissue represents an artifact. An erroneous signal caused by contaminating leukocytes is unlikely for the following reasons. First, as discussed above, our microscopic studies show that both P-selectin and PSGL-1 peptide antibody specifically bind to membranous structures within the zona pellucida. Second, large amounts of leukocytes (~0.5 mg proteins of membrane extract from ~100 million leukocytes per lane) are typically required for positive detection with the P-selectin blotting method used in this report (Fig. 1 *D*; Ma et al., 1994). Hence, if the molecule detected in the zona pellucida represents the leukocyte ligand, truly major leukocyte contaminations of the zona pellucida preparations would be required to produce the signal observed on the blots. However, light and EM examination of these preparations failed to show such leukocyte presence. Third, nonspecific binding of P-selectin and PSGL-1 peptide antibody to a contaminating or otherwise unrelated polysaccharide and/or protein structure(s) on the surface of the membranous structures in the zona pellucida is unlikely since our blotting experiments clearly show that both P-selectin and PSGL-1 peptide antibody primarily recognize one protein among the considerable numbers of proteins present in the zona preparation; in addition, this protein is one of the minor constituents of the zona pellucida (Fig. 1, compare *A*, *B*, and *C*). Finally, the expression of a P-selectin ligand in the zona pellucida is consistent with the detection of PSGL-1 mRNA in the mouse ovary, as demonstrated by Northern analysis (Yang et al., 1996).

The P-selectin ligand from porcine zona pellucida and leukocytes described in this report shares many characteristics with human leukocyte PSGL-1 (Moore et al., 1992; Sako et al., 1993; Lenter et al., 1994; Ma et al., 1994; Asa et al., 1995). These include biochemical properties (disulfide-linked dimeric protein), functional properties (recognition by P-selectin in a Ca^{2+}-dependent manner), and polypeptide properties (recognition by PSGL-1 peptide antibody). However, there is a difference in molecular masses between the human leukocyte PSGL-1 (~240 kD under nonreducing conditions and ~100 kD under reducing conditions) (Ma et al., 1994) and the porcine oocyte and leukocyte P-selectin ligands (~210 kD under nonreducing conditions and ~80 kD under reducing conditions) (Figs. 1 and 2). Therefore, since amino acid sequencing information is not available for the porcine oocyte P-selectin ligand, the question as to whether this molecule is identical to PSGL-1 remains to be answered.

In this study we failed to detect any expression of E-selectin on sperm cells by flow cytometry, immunoblotting, or immunogold EM. The apparent absence of the expression of E-selectin on porcine sperm cells argues against a biological role for this lectin in gamete interactions. However, an immunohistologic study has demonstrated the expression of both E- and P-selectin on vascular endothelial cells in the decidua basalis, but not on decidua parietalis (Burrows et al., 1994). Therefore, it is conceivable that E-selectin, along with P-selectin, on vascular endothelial cells in the decidua basalis may interact with the zona pellucida ligand during trophoblast implantation.

The expression of P-selectin on the acrosomal membrane of porcine sperm cells is supported by several experimental findings. First, blotting of sperm cell extracts separated on SDS-PAGE with two well-characterized P-selectin antibodies (Ma et al., 1994; Asa et al., 1995; Toombs et al., 1995) resulted, for both reagents, in the detection of a protein band with an apparent molecular mass identical to those of human platelet P-selectin (Ma et al., 1994) and porcine platelet P-selectin (Fig. 7). Since purified sperm cells (see Materials and Methods) were used for this experiment, it is unlikely that platelet contamination is responsible for the signal on the blot; as argued above, a considerable amount of platelets are required for a positive signal in this type of experiment. Second, immunoelectron microscopies of the porcine sperm cells quite unequivocally demonstrate the abundant presence of a molecule recognized by a P-selectin antibody, on what appears to be the acrosomal membrane of the sperm cells (Fig. 8). This distribution to a specific membranous compartment on the sperm cells is in itself an argument against nonspecific labeling, caused by unspecific binding of the antibody as well as by unspecific adsorption of (soluble) antigen to the sperm cell surface. The fact that only acrosome-reacted sperm cells are capable of binding the antibodies also argues against nonspecific results. Finally, the capacity of acrosome-reacted porcine sperm cells to attach to oocytes and HL-60 cells in a manner that is inhibitable by P-selectin antibodies strongly suggests a P-selectin function on the acrosome-reacted sperm cells (Table I; Fig. 9).

The ultrastructural distribution of the zona pellucida P-selectin ligand indicates that it is less likely a component of the zona pellucida matrix. Instead, the molecule appears to be located exclusively in membranes embedded within the matrix of the zona pellucida. The membrane structures in the zona pellucida may originate from the long oocyte microvilli and/or from the follicle cell projections that transverse the zona pellucida and make contact (gap junctions) with the oocyte plasma membrane (Austin, 1968). The morphological localization of P-selectin ligand–containing membranes in the zona pellucida implicates a potentially functional role for a P-selectin ligand during

the penetration by the acrosome-reacted sperm cells through the thick, gel-like matrix of the zona pellucida. In this regard, the expression of functional P-selectin on the acrosomal membrane of sperm cells supports this hypothesis.

The findings in this report implicate a potentially biological role for the zona pellucida P-selectin ligand and the sperm P-selectin in porcine gamete interactions. However, since homozygous mice deficient in P-, E-, or L-selectin, by homologous recombination, have no apparent deficiencies in breeding (Myadas et al., 1993; Arbonès et al., 1994; Labow et al., 1994), this interaction may be only one of several molecular mechanisms involved in fertilization in vivo (Wassarman, 1995; Snell and White, 1996). A similar, apparently redundant pathway has been described for leukocyte-endothelial cell interactions. The phenotypes of P-, E-, and L-selectin knockout mice appear normal until the animals are challenged by inflammatory mediators (Myadas et al., 1993; Arbonès et al., 1994; Labow et al., 1994).

Taken together, our in vitro studies suggest that a mechanism, similar to that involved in leukocyte recruitment, may be involved in sperm–egg binding. However, the specific role(s) of these molecules in the different steps of sperm–egg interaction, especially in vivo, clearly require(s) further investigation.

We thank Drs. C.W. Smith for peptide synthesis and M.R. Deibel for detailed protocol of affinity isolation of PSGL-1 peptide Ab on immobilized peptide beads. We are grateful to Drs. S. Kornfeld and A.E. Buhl for critical comments on the manuscript. We also thank P.A. Aeed, R.A. Evans, and D.D. Gleason for their technical expertise during this study.

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PRECEDENTIAL OPINION

Pursuant to the Board of Patent Appeals and Interference's Standard Operating Procedure 2, the opinion below has been designated a precedential opinion.

UNITED STATES PATENT AND TRADEMARK OFFICE

BEFORE THE BOARD OF PATENT APPEALS
AND INTERFERENCES

Ex parte MAREK Z. KUBIN and RAYMOND G. GOODWIN

Appeal 2007-0819
Application 09/667,859
Technology Center 1600

Decided: May 31, 2007

Before MICHAEL R. FLEMING, *Chief Administrative Patent Judge,* TEDDY S. GRON, TONI R. SCHEINER, ERIC GRIMES, and NANCY J. LINCK, *Administrative Patent Judges.*

LINCK, *Administrative Patent Judge.*

DECISION ON APPEAL

This is a 35 U.S.C. § 134 appeal in the above-referenced case.[1]

We have jurisdiction under 35 U.S.C. § 6(b). We affirm.

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[1] The application was filed September 20, 2000. The real party in interest is Immunex Corporation, a wholly owned subsidiary of Amgen Inc.

STATEMENT OF THE CASE

The field of the invention is polynucleotides encoding NK (natural killer) Cell Activation Inducing Ligand ("NAIL") polypeptides. (Specification ("Spec.") 1.) NK cells play a role in the "early, innate immune system" and "appear to be closely related to T cells." (*Id.*) Like T cells, the immune response of NK cells "involves direct cytotoxicity and production of various cytokines" that stimulate the immune system. (*Id.* at 3.)

"NK cells have been implicated as mediators of host defenses against infection in humans with varicella zoster, herpes simplex, cytomegalovirus, Epstein-Barr virus, hepatitis B, and hepatitis C viruses." (*Id.* at 3.) They also are "involved in both resistance to and control of cancer spread," including leukemia (*id.* at 3) and "play a . . . role in bone marrow transplant rejection, as well as solid organ transplant rejection." (*Id.* at 4.) Thus, "depletion of NK cells can result in a decreased resistance to target tissue infection by viruses." (*Id.* at 2.) Finally, "a number of human lymphoproliferative disorders of NK cells are known." (*Id.*) "With the function of NK cells so important in this variety of physiological responses, there is a need in the art for methods of controlling NK function." (*Id.*)

NAIL is a cell surface marker, or receptor, on the surface of NK cells that modulates the activity of NK cells. (*See id.* at 2.) Thus, modulation of NAIL activity would be expected to modulate NK cell function, thereby stimulating or inhibiting the immune response.

"CD48 is a membrane glycoprotein found on cells of hematopoietic origin." (*Id.* at 6.) "cDNA clones for CD48 have been isolated" and the

"nucleotide and amino acid sequences of CD48 are known." (*Id.*) Antibodies to CD48 appear to suppress cell mediated immunity. (*Id.* at 6-7.) "The identification of CD48 as a NAIL counter-structure . . . allows the generation of molecules that can modulate the activation of NK . . . cells." (*Id.* at 45.) Thus, the determination of binding to CD48 potentially provides a useful tool to identify active variants of NAIL. (*See, e.g.*, claim 73.)

The claimed subject matter is reflected in representative claim 73:[2]

73. An isolated nucleic acid molecule comprising a polynucleotide encoding a polypeptide at least 80% identical to amino acids 22-221 of SEQ ID NO:2, wherein the polypeptide binds CD48.

The Examiner has rejected claims 73-78 and 80-89 under 35 U.S.C. § 103(a) over the combined teachings of Valiante et al., U.S. Patent No. 5,688,690 (issued Nov. 18, 1997) ("Valiante"); Sambrook et al., *Molecular Cloning: A Laboratory Manual*, 2nd Edition, 2.43-2.84 (Cold Spring Harbor, N.Y. 1989) ("Sambrook");[3] and Porunelloor Mathew et al., *Cloning and Characterization of the 2B4 Gene Encoding a Molecule Associated with Non-MHC-Restricted Killing Mediated by Activated Natural Killer Cells and T Cells*, 151 J. IMMUNOL., 5328-5337 (1993) ("Mathew").[4]

The Examiner also has rejected claims 73, 74, 80, and 84-89 under 35 U.S.C. § 112, ¶ 1, for lack of enablement and written description.

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[2] Appellants do not separately argue the claims. Thus, we address each issue with reference to claim 73.

[3] We note Sambrook is incorporated by reference in Valiante (col. 7, ll. 55-57).

[4] This reference is referred to as "Porunelloor" by the Examiner and Appellants.

OBVIOUSNESS UNDER § 103(a)

The § 103(a) Issue

The Examiner contends the skilled artisan would have been motivated to isolate the nucleic acid sequence corresponding to NAIL, based on Valiante's disclosure of p38 (which is the same protein as NAIL) and Valiante's express teachings how to isolate p38 cDNA by using conventional techniques, such as taught in Sambrook, including using mAb C1.7, a probe specific for p38. (Answer 11-16.)

Appellants contend: "As in *Deuel*, it is not proper for the Office to use the p38 protein identified in the '690 patent [Valiante] together with the methods such as those described in Sambrook et al. to reject claims drawn to specific sequences." (Br. 19 (citing *In re Deuel*, 51 F.3d 1552, 34 USPQ2d 1210 (Fed. Cir. 1995).)

We frame the § 103(a) issue: Would Appellants' claimed nucleotide sequence have been obvious to one of ordinary skill in the art, based on Valiante's disclosure of p38 and his express teachings how to isolate its cDNA by conventional techniques?

Findings of Fact Relating to Obviousness

1. Valiante's p38 protein is a 38kd molecule recognized by mAb C1.7, and is the same protein as Appellants' NAIL, "formerly known as C1.7." (Spec. 10: 29-30. *See also* Answer 14; Spec. 11: 4.)

2. Valiante expressly teaches through a prophetic example how to "isolat[e] the cDNA clone by using [mAb] C1.7, screening the protein expression in the cell transfected with the cDNA library and cloning a corresponding cDNA into a plasmid for sequencing." (Answer 12 (citing Valiante, col. 7, l. 48 through col. 8, l. 7 & example 12, cols. 18-19).)

3. Valiante does not disclose the sequence of p38 recognized by mAb C1.7 or the DNA encoding p38. (*See* Valiante passim; Answer 12.)

4. The DNA and protein sequences of p38, and thus NAIL, could have been obtained by conventional methodologies, such as those taught by Sambrook. (Valiante, col. 7, l. 48 to col. 8, l. 7; *see also* Answer 12.)

5. Sambrook is incorporated by reference in Valiante. (Col. 7, ll. 55-57.)

6. Mathews' cell surface signaling molecule, 2B4, is the mouse version of Valiante's p38, the human version. (Answer 15.)

7. Mathews cloned the gene encoding 2B4 and determined its nucleotide sequence. (Mathews at 5328 (Abstract).)

8. The relevant teachings in Mathews are cumulative to the teachings in Valiante and Sambrook and merely are exemplary of how routine skill in the art can be utilized to clone and sequence the cDNA of a similar polypeptide. (*See* Answer 15.)

9. Appellants employed conventional methods, "such as those outlined in Sambrook," to isolate a cDNA encoding NAIL and determine the cDNA's full nucleotide sequence (SEQ NOS: 1 & 3). (Spec. 10: 29 to 13: 7; Spec. 16: 40 to 17: 1; Spec. 65 (Example 1).)

10. Appellants' claimed polynucleotide is "isolated from [a] cDNA library . . . using the commercial monoclonal antibody C1.7 . . . disclosed by Valiante." (Answer 13. *See also* Spec. 65: 17-32.)

11. As acknowledged by Appellants, "the level of skill in the art is high." (Br. 11 (citing *In re Wands*, 858 F.2d 731, 740, 8 USPQ2d 1400, 1404 (Fed. Cir. 1988).)

12. The state of the art had unquestionably advanced significantly during the ten year period between the time the *Deuel* application was filed in 1990 and Appellants' application was filed in 2000. *See In re Wallach*, 378 F.3d 1330, 1333, 71 USPQ2d 1939, 1942 (Fed. Cir. 2004).

13. As acknowledged by Appellants, "methods of making the claimed nucleic acid sequences . . . are known." (Br. 11 (citing *Wands*, 858 F.2d at 740). *See also* Br. 3 ("isolation of clones is well known in the art").)

14. Valiante's disclosure of the polypeptide p38, and a detailed method of isolating its DNA, including disclosure of a specific probe to do so, i.e., mAb C1.7, established Valiante's possession of p38's amino acid sequence and provided a reasonable expectation of success in obtaining a polynucleotide encoding p38, a polynucleotide within the scope of Appellants' claim 73. (*See* Valiante, col. 7, l. 48 to col. 8, l. 7.)

15. As recently clarified by the Federal Circuit, possession of the cDNA encoding NAIL also provided possession of its nucleic acid sequence, i.e., "its identity." *In re Crish*, 393 F.3d 1253, 1258, 73 USPQ2d 1364, 1368 (Fed. Cir. 2004).

16. One of ordinary skill in the art would have had a reasonable likelihood of success that he or she would have been able to obtain the nucleotide encoding NAIL using conventional methods, such as disclosed in Valiante. (*See* col. 7, l. 48 to col. 8, l. 33.)

17. NAIL is "a signal transduction surface molecule (p38) expressed by virtually all human NK cells" and thus plays a role in the immune response. (Valiante, col. 2, l. 65 to col. 3, l. 40.)

18. Thus, one of ordinary skill in the art would have recognized the value of isolating NAIL cDNA, and would have been motivated to apply

conventional methodologies, such as those disclosed in Sambrook and utilized in Valiante, to do so. (*See, e.g.*, Valiante, col. 7, l. 48 to col. 8, l. 33.) *See Alza Corp. v. Mylan Labs*, 464 F.3d 1286, 1289, 80 USPQ2d 1001, 1003 (Fed. Cir. 2006) ("'The presence or absence of a motivation to combine references in an obviousness determination is a pure question of fact.' *In re Gartside*, 203 F.3d 1305, 1316, 53 USPQ2d 1769, 1776 (Fed. Cir. 2000).").

Discussion of the § 103(a) Issue

Based on our findings and those of the Examiner, at least one of Appellants' claimed polynucleotides would have been obvious to one of ordinary skill in the art at the time Appellants' invention was made. Regardless of some factual similarities between *Deuel* and this case, *Deuel* is not controlling and thus does not stand in the way of our conclusion, given the increased level of skill in the art and the factual differences. *See In re Wallach*, 378 F.3d 1330, 1334, 71 USPQ2d 1939, 1942 (Fed. Cir. 2004) ("state of the art has developed [since] *In re Deuel*").

Appellants argue the "cited references do not provide an adequate written description of the claimed nucleic acid sequences." (Reply Br. 18 (citing *Noelle v. Lederman*, 355 F.3d 1343, 69 USPQ2d 1508 (Fed. Cir. 2004)). In so arguing, Appellants overlook the distinction between obviousness under § 103 and lack of written description under § 112, § 1. A single, obvious species within a claimed genus renders the claimed genus unpatentable under § 103. Thus, an obvious method of obtaining a single nucleic acid molecule encoding NAIL may be all that is required to show that the presently claimed genus of nucleic acid molecules is unpatentable under § 103. In contrast, as discussed *infra* (see pp. 15-17), the description

of a single species within a claimed genus may not be sufficient to support the patentability of the genus under § 112, ¶ 1. *See University of California v. Eli Lilly & Co.*, 119 F.3d 1559, 1567, 43 USPQ2d 1398, 1405 (Fed. Cir. 1997) (noting the court earlier held "a description which renders obvious a claimed invention is not sufficient to satisfy the written description requirement of that invention" and in this case holding disclosure of a species did not provide adequate written description of a genus). *Cf. Eli Lilly & Co. v. Barr Labs*, 251 F.2d 955, 971, 58 USPQ2d 1869, 1880 (Fed. Cir. 2001) ("later genus claim limitation is anticipated by, and therefore not patentably distinct from, an earlier species claim").

With respect to the written description requirement, while "examples explicitly covering the full scope of the claim language" typically will not be required, a sufficient number of representative species must be included "to demonstrate that the patentee possessed the full scope of the [claimed] invention." *Lizardtech, Inc. v. Earth Resource Mapping, Inc.*, 424 F.3d 1336, 1345, 76 USPQ2d 1724, 1732 (Fed. Cir. 2005). Thus, Appellants' argument based on alleged lack of written description in the cited prior art is unavailing.

Appellants heavily rely on *Deuel*. (*See, e.g.*, Br. 19.) To the extent *Deuel* is considered relevant to this case, we note the Supreme Court recently cast doubt on the viability of *Deuel* to the extent the Federal Circuit rejected an "obvious to try" test. *See KSR Int'l Co. v. Teleflex Inc.*, 127 S.Ct. 1727, ___, 82 USPQ2d 1385, 1394, 1396 (2007) (citing *Deuel*, 51 F.3d at 1559). Under *KSR*, it's now apparent "obvious to try" may be an appropriate test in more situations than we previously contemplated.

When there is motivation

> to solve a problem and there are a finite number of identified, predictable solutions, a person of ordinary skill has good reason to pursue the known options within his or her technical grasp. If this leads to anticipated success, it is likely the product not of innovation but of ordinary skill and common sense. In that instance the fact that a combination was obvious to try might show that it was obvious under § 103.

KSR Int'l Co. v. Teleflex Inc., 127 S. Ct. 1727, ___, 82 USPQ2d 1385, 1397 (2007). This reasoning is applicable here. The "problem" facing those in the art was to isolate NAIL cDNA, and there were a limited number of methodologies available to do so. The skilled artisan would have had reason to try these methodologies with the reasonable expectation that at least one would be successful. Thus, isolating NAIL cDNA was "the product not of innovation but of ordinary skill and common sense," leading us to conclude NAIL cDNA is not patentable as it would have been obvious to isolate it.

Appellants also argue lack of motivation to combine the cited references. (Br. 20-22; Reply Br. 19-21.) Motivation to combine references "may be found in implicit factors, such as 'knowledge of one of ordinary skill in the art, and [what] the nature of the problem to be solved as a whole would have suggested to those of ordinary skill in the art'." *Alza Corp. v. Mylan Labs.*, 464 F.3d 1286, 1291, 80 USPQ2d 1001, 1004 (Fed. Cir. 2006) (quoting *In re Kahn*, 441 F.3d 977, 988, 78 USPQ2d 1329, 1337 (Fed. Cir. 2006)). *See also KSR*, 127 S. Ct. at ___, 82 USPQ2d at 1396 (citing with approval *In re Kahn*, 441 F.3d at 988, 78 USPQ2d at 1336).

More specifically, Appellants argue Mathews "teaches that a human homolog is not expressed," and thus "a person of skill in the art would not be motivated to combine" Mathews with Valiante. (Reply Br. 21.)

Appellants support this argument by quoting from Mathews: "Genomic Southern blots identified a human homologue of the 2B4 gene. However, RNA blot analysis of total RNA isolated from human NK cells suggests that 2B4 gene is not expressed in humans." (Mathews, at 5333, col. 1.)

Rather than teaching away from the combination, as Appellants argue, this language merely indicates conflicting data existed regarding a 2B4 homolog in humans, with some data pointing to the existence of a human homolog. (*See id.*) The quoted language would not have deterred the skilled artisan from obtaining the cDNA corresponding to Valiante's p38, as taught by Valiante, i.e., "from following the path set out in the reference." *In re Gurley*, 27 F.3d 551, 553, 31 USPQ2d 1130, 1131 (Fed. Cir. 1994), *quoted with approval in In re Kahn*, 441 F.3d 977, 990, 78 USPQ2d 1329, 1338 (Fed. Cir. 2006). Moreover, Appellants miscomprehend the value of Mathews. Mathews exemplifies how the cDNA encoding 2B4, the mouse version of Valiante's p38 expressed on all NK cells, can be isolated and sequenced. (*See* Mathews at 5328 (Abstract).) Thus, the teachings of Mathews, when considered as a whole, support the Examiner's § 103 ground of rejection.

PATENTABILITY UNDER § 112, ¶ 1

The Enablement Issue

The Examiner found lack of enablement due to the "at least 80% identity language," in the absence of any working examples, other than SEQ ID NOS:1 and 2. He cites examples in the literature in which very small changes in sequence, even one amino acid, yield a different function. (Answer 3-6.)

Appellants respond: "The Office's reasoning ignores the many references that positively demonstrate that proteins can be mutated and maintain a biological function." (Reply Br. 4 (citing numerous publications in support).) Moreover, "the specification provides extensive guidance for creating and screening mutants" (Reply Br. 5) in that it "teaches in detail how to: 1) make variants of SEQ ID NOs: 1 and 2; 2) calculate the percent identity between SEQ ID NOs: 1 and 2 and the variant sequence; and 3) test the variant sequence to determine if it binds to CD48" (Br. 11; Reply Br. 6). Thus, according to Appellants, only routine experimentation would be required to practice the claimed invention. (Reply Br. 9.)

In view of these conflicting positions, we frame the enablement issue as follows: Considering the relevant *Wands* factors, including the prior art teachings cited by the Examiner and Appellants to establish the level of predictability in the relevant art, would undue experimentation have been required to practice the full scope of claim 73?

The Written Description Issue

The Examiner bases his written description rejection on the same claim language as the enablement rejection, i.e., "at least 80% identity," and finds Appellants' disclosed sequences inadequate to show "possession of" their claimed genus. (Answer 9 (citing *University of California v. Eli Lilly & Co.*, 119 F.3d 1559, 43 USPQ2d 1398 (Fed. Cir. 1997).)

In response, Appellants contend (1) *Lilly* can be distinguished on its facts and (2) the Examiner's position is inconsistent with Example 14 in the

Office's "Synopsis of Application of Written Description Guidelines"[5] (hereafter "*Synopsis*") (www.uspto.gov/web/patents/guides.htm), an example which contains "analysis of [a] claim that is highly similar to the claims at issue." (Reply Br. 13.)

In view of the above, we frame the written description issue: Does Appellants' Specification contain a written description sufficient to show they had possession of the full scope of their claimed invention at the time the application was filed, as required by Federal Circuit precedent?

Findings of Fact Relating to § 112, ¶ 1

19. Claim 73 is limited to isolated polynucleotides encoding polypeptides (1) which are "at least 80% identical to amino acids 22-221 of SEQ ID NO:2" (the amino acid sequence for the extracellular domain of NAIL without the signal sequence), and (2) which bind CD48. (*See* claim 73; Spec. 13: 9-18.)

20. The Specification provides two working examples within the scope of claim 73, i.e., a DNA encoding NAIL (SEQ ID NO: 1) and NAIL's coding sequence with accompanying upstream and downstream noncoding sequences (SEQ ID NO: 3). SEQ ID NOs: 1 and 3 both encode the amino acid sequence of SEQ ID NO: 2. (Spec. 10: 29 to 13: 7; Spec. 16: 40 to 17: 1; Spec. 65 (Example 1).)

21. The Specification also discloses the amino acid sequences for three fusion proteins (SEQ ID NOs: 6, 7, & 8) whose nucleotide sequences would fall within the scope of claim 73. (Spec. 25: 30 to 33: 9.)

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[5] Guidelines for Examination of Patent Applications Under the 35 U.S.C. 112, ¶ 1 "Written Description" Requirement, 66 Fed. Reg. 1099 (Jan.5, 2001) ("*Written Description Guidelines*").

22. The Specification does not disclose any variants in which the nucleotide sequence encoding amino acids 22-221 of SEQ ID NO:2 is varied. (Spec. *passim.*)

23. Thus, the Specification does not disclose "which 20% . . . of amino acid residues should be changed in order to maintain the biological functions for binding to CD48." (Answer 5.)

24. The Specification "teaches in detail how to: 1) make variants of SEQ ID NOs: 1 and 2; 2) calculate the percent identity between SEQ ID NOs: 1 and 2 and the variant sequence; and 3) test the variant sequence to determine if it binds to CD48." (Br. 11; Reply Br. 6.)

25. The Specification does not disclose a correlation between function (binding to CD48) and structure responsible for binding to CD48 (other than the entire extracellular domain) such that the skilled artisan would have known what modifications could be made of the very large number of modifications potentially encompassed by claim 73 without losing function. (*See* Spec. *passim*; Answer 10.)

26. At the time Appellants' application was filed, molecular biology was generally an unpredictable art, as evidenced by the references cited by the Examiner. (Answer 4 (citing Robin E. Callard & Andy J.H. Gearing, The Cytokine FactsBook 188-89 (Academic Press 1994); Struyf et al., *Natural truncation of RANTES abolishes signaling through the CC chemokine receptors CCR1 and CCR3, impairs its chemotactic potency and generates a CC chemokine inhibitor*, 28 Eur. J. Immunol. 1262-71 (1998); & Proudfoot et al., U.S. Patent 6,159,711 (Dec. 12, 2000).)

27. At the time Appellants' application was filed, the level of skill in the relevant art (molecular biology) was high, as acknowledged by Appellants. (Br. 11.)

28. "[M]ethods of making the claimed nucleic acid sequences and screening for activity [were] known in the art and described in the specification." (Br. 11-12.)

29. The "experimentation involved to produce other sequences within the scope of the claims" and thus to practice the full scope of claim 73, would have been "well within the skill of those in the art" (Br. 12) and thus would have been routine.

30. One of ordinary skill in the art would not have been required to perform undue experimentation to practice the invention of claim 73.

Discussion of the Enablement Issue

In making the above findings, we have considered the relevant *Wands* factors in light of the prior art teachings relied upon by the Examiner and Appellants, and the relevant caselaw. We agree with the Examiner that molecular biology is generally an unpredictable art (and thus was so at the time the application was filed). However, with respect to enablement, the other *Wands* factors weigh in Appellants' favor, particularly "the state of the art" and "the relative skill of those in the art," *In re Wands*, 858 F.2d 731, 736, 8 USPQ2d 1400, 1404 (Fed. Cir. 1988), as evidenced by the prior art teachings and Appellants' Specification.

The amount of experimentation to practice the full scope of the claimed invention might have been extensive, but it would have been routine. The techniques necessary to do so were well known to those skilled in the art. *See, e.g., Johns Hopkins Univ. v. Cellpro, Inc.*, 152 F.3d 1342,

1360, 47 USPQ2d 1705, 1719 (Fed. Cir. 1998) ("test [for undue experimentation] is not merely quantitative . . . if it is merely routine"). A "patent need not teach, and preferably omits, what is well known in the art." *Hybritech Inc. v. Monoclonal Antibodies, Inc.*, 802 F.2d 1367, 1384, 231 USPQ 81, 94 (Fed. Cir. 1986). Thus, we conclude the Specification would have enabled the full scope of claim 73.

Discussion of the Written Description Issue

In spite of concluding claim 73 would have been enabled, Federal Circuit caselaw compels us to find the written description requirement is not met. *See generally, e.g., University of Rochester v. G.D. Searle & Co.*, 358 F.3d 916, 69 USPQ2d 1886 (Fed. Cir. 2004); *Enzo Biochem. Inc. v. Gen-Probe Inc.*, 323 F.3d 956, 63 USPQ2d 1609 (Fed. Cir. 2002); *University of California v. Eli Lilly & Co.*, 119 F.3d 1559, 43 USPQ2d 1398 (Fed. Cir. 1997); *Fiers v. Revel*, 984 F.2d 1164, 25 USPQ2d 1601 (Fed. Cir. 1993).

"Although there is often significant overlap" between the enablement and written description requirements, "they are nonetheless independent of each other." *University of Rochester*, 358 F.3d at 921, 69 USPQ2d at 1891. An "invention may be enabled even though it has not been described." *Id.* Such is the situation here. While we conclude one skilled in the art would have been able to make and use the full scope of claim 73 through routine experimentation, we find Appellants did not describe the invention of claim 73 sufficiently to show they had possession of the claimed genus of nucleic acids. *See, e.g., Noelle v. Lederman*, 355 F.3d 1343, 1348, 69 USPQ2d 1508, 1513 (Fed. Cir. 2004) ("invention is, for purposes of the 'written description' inquiry, *whatever is now claimed*").

Claim 73 is to a genus of polynucleotides encoding polypeptides "at least 80% identical to amino acids 22-221 of SEQ ID NO:2" which bind to CD48. Sufficient description to show possession of such a genus "may be achieved by means of a recitation of a representative number of cDNAs, defined by nucleotide sequence, falling within the scope of the genus or of a recitation of structural features common to members of the genus, which features constitute a substantial portion of the genus." *Eli Lilly*, 119 F.3d at 1568, 43 USPQ2d at 1406. Possession may not be shown by merely describing how to obtain possession of members of the claimed genus or how to identify their common structural features. *See University of Rochester*, 358 F.3d at 927, 69 USPQ2d at 1895.

In this case, Appellants have sequenced two nucleic acids falling within the scope of claim 73 and three fusion proteins whose nucleotide sequences would fall within the scope of claim 73. None of these sequences varies amino acids 22-221 of NAIL, and thus these sequences are not representative of the genus.

Appellants also have described how to make and test other sequences within claim 73 sufficiently to satisfy the enablement requirement. However, they have not described what domains of those sequences are correlated with the required binding to CD48, and thus have not described which of NAIL's amino acids can be varied and still maintain binding. Thus, under *Lilly* and its progeny, their Specification would not have shown possession of a sufficient number of sequences falling within their potentially large genus to establish possession of their claimed genus. *Cf. Enzo*, 323 F.3d at 964, 63 USPQ2d at 1612 ("if the functional characteristic of . . . binding to [CD48] were coupled with a disclosed correlation between

that function and a structure that is sufficiently known or disclosed," the written description requirement may be met).

Without a correlation between structure and function, the claim does little more than define the claimed invention by function. That is not sufficient to satisfy the written description requirement. *See Eli Lilly*, 119 F.3d at 1568, 43 USPQ2d at 1406 ("definition by function ... does not suffice to define the genus because it is only an indication of what the gene does, rather than what it is").

With respect to Appellants' reliance on hypothetical Example 14 in the Office's *Synopsis*, "[c]ompliance with the written description requirement is essentially a fact-based inquiry that will 'necessarily vary depending on the nature of the invention claimed.'" *Vas-Cath Inc. v. Mahurkar*, 935 F.2d 1555, 1563, 19 USPQ2d 1111, 1117 (Fed. Cir. 1991) (quoting *In re DiLeone*, 436 F.2d 1404, 1405, 168 USPQ 592, 593 (CCPA 1971)), *quoted with approval in Enzo*, 323 F.3d at 963, 63 USPQ2d at 1612. While the *Written Description Guidelines* and the hypothetical examples in the Office's *Synopsis* can be helpful in understanding how to apply the relevant law (as it existed in 2001 when the Guidelines were adopted), they do not create a rigid test.

Based on the above, we find the written description requirement of § 112, ¶ 1, is not met.

CONCLUSION

In summary, with respect to claim 73, we affirm the § 103(a) rejection, reverse the § 112, ¶ 1, enablement rejection, and affirm the § 112, ¶ 1, written description rejection.

Pursuant to § 41.37(c)(1)(vii)(2006), we also affirm the rejection of claims 74-78 and 80-89 under § 103(a); reverse the § 112, ¶ 1, enablement rejection of claims 74, 80, and 84-89; and affirm the § 112, ¶ 1, written description rejection of claims 74, 80, and 84-89, as these claims were not argued separately.

No time period for taking any subsequent action in connection with this appeal may be extended under 37 C.F.R. § 1.136(a)(1)(iv)(2006).

AFFIRMED

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